BIOCHEMICAL GENETIC STUDIES OF MAIZE PEROXIDASES

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ABSTRACT

Genetic and biochemical properties of principal peroxidases of maize, Px3 and Px7, were determined and their changes during development of morphological mutants were examined. Horizontal gel electrophoresis was applied for resolution of peroxidase isozyme bands.

An unusual Px3 allele, Px3-6, produced two bands electrophoresing similar to those produced by alleles Px3-1 and Px3-2. A hypothesis was tested that Px3-6 is a gene duplication. Densitometric methods identified the S and F bands of Px3-6 to be electrophoretically identical to those of Px3-1(S) and Px3-2(F). Electrophoresis under varying gel concentrations showed no evidence of differences in mobility and size between S and F bands from Px3-6($\overline{SF}$), Px3-1(S) and PX3-2(F) alleles. Genetic results suggested that Px3-6 ($\overline{SF}$) allele is a tandem duplication of Px3 genes.

Chromosomal location of Px3 locus previously assigned to Chromosome 7, was determined using gene markers o_2, Pn, sl and Tp, and waxy translocation 7-9a. The most probable location of Px3 was assigned between 96.6 and 100.32 on the long arm of Chromosome 7.

Partial purification of enzymes Px3 and Px7 was achieved by $(NH_4)_2SO_4$ fractionation, Con A chromatography and Blue Sepharose chromatography. Anodal and catho peroxidases could be separated by Con A chromatography, and Px3 and Px7 were separated by Blue Sepharose.

Michaelis constant (Km) values of Px3 and Px7 were determined for six allelic enzymes of the two loci. No significant differences were

found in Km values among allozymes at each locus on these substrates--ferulic acid, caffeic acid and o-dianisidine. However, Km values of Px3 and Px7 allozymes on ferulic acid were significantly different. Px7 showed higher affinity to ferulic acid, a lignin precursor, evidently utilize ferulic acid more efficiently than Px3 and make a greater contribution of lignin formation in maize plants. The molecular size of Px7 was shown to be 72,000, about twice as much as large as Px3 (MW35,000). Genetic studies, however, provided no evidence that Px7 produces dimeric or polymeric proteins.

Studies were conducted on peroxidase variations during the pro-flowering development of 14 isogenic, morphological mutants of maize. Leaf peroxidase activity increased during development (4-7 weeks) of five non-GA responding dwarf mutants--br, br2, na, and py--, while two GA-responding dwarfs, d and d-tn had the same level of peroxidase activity as control. Except for na2, no change in band pattern was found. Other types of morphological mutants, Knotted (Kn), slashed leaf (sl) and Ragged leaf (Rg) also showed high peroxidase activity relative to control at the later stages of development (5-7 weeks). Increasing peroxidase activity in mutant Rg was characterized by an isozyme previously unreported, and designated Px-Rg. Increased peroxidase activity may relate to elevated lignin formation or phenol oxidation in 'diseased-like' mutant plant Rg.

Wind effects on young maize plants were examined under continuous wind (1.8m/s - 4.2m/s velocity) in greenhouse experiments. After 3 days of treatment, retardation in plant growth (plant height, fresh

weight) of 4.2m/s wind treated plants was observed. After 7 days of treatment, peroxidase activity was significantly higher than that of control. It was clear that wind (4.2m/s) affected plants both in retardation in growth and elevated peroxidase activity.

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I. INTRODUCTION

Peroxidases (donor: H_2O_2 oxidoreductase: EC 1.11.1.7) are enzymes that occur widely and are highly polymorphic in plant tissues. Peroxidases perform several major developmental roles in plants, but these have not been completely clarified. Probable peroxidase roles include auxin degradation, lignin synthesis and oxidation of phenolic compounds. Peroxidase level in plants is reported to increase in response to environmental stress conditions including mechanical injuries. Disease-affected tissues and certain morphological mutants, notably dwarfs, are also known to have unusually high peroxidase activity (Stahman et al. 1966, Price and Stebbins 1971).

Biochemical properties are known for peroxidases from plants such as horseradish and turnip (Shannon et al. 1968, Welinder and Mazza 1977). Characterization of these isozymes has shown that each is different in its substrate specificity (Srivastava et al. 1977), suggesting that each isozyme has a specific physiological role in plant tissue. On the other hand, tissue and ontogenetic polymorphisms of peroxidases (Gordon 1971, Rychter and Lewak 1971) implicate them in development and differentiation of plants. If a specific gene for a peroxidase "turns on" at a specific developmental stage, the specific isozyme may function in a precise developmental role in growth. One of the approaches to clarify this mechanism of plant development is to investigate the relationship of gene, enzyme and physiological role. Few studies of this type have been conducted, due in part to the fact

that most plant peroxidase studies have utilized materials in which the genetics are unknown (e.g., horseradish).

Maize is one of the best plant materials for this purpose since it has received intensive genetic study and its genetic control mechanisms are relatively well understood (Coe and Neuffer 1977). In maize, 9 loci which govern peroxidase polymorphisms with a total 26 alleles, have been identified in Hawaii (Brewbaker and Hasegawa 1975). Tissue and ontogenetic polymorphisms have also been reported (Hamill 1970, Hasegawa 1974).

The objectives of this study were to investigate the relationship between genetic and biochemical properties of the principal maize peroxidase isozymes and their physiological roles in developmental stages of maize. The study included the characterization of principal loci Px3 and Px7, and biochemical studies of allozymes for these loci. Peroxidase variations during the development of morphological mutants of maize were also examined, in part to assess the timing of gene action of mutant phenotypes. The effects of wind stress on plant growth and peroxidases were studied, and clues sought to define the physiological role of peroxidases under conditions of environmental stress.

II. LITERATURE REVIEW

2.1 Peroxidase: Biochemical Characteristics

2.1.1 Chemical Structure and Characteristics.

Peroxidases (EC 1.11.1.7: Donor: H_2O_2 oxidoreductase) are enzymes defined by catalytic ability to oxidize various hydrogen donors in the presence of H_2O_2 or other peroxidases. Wide distribution of peroxidases has been found in plants, animals, fungi and bacteria (Saunders et al. 1964). Peroxidases are commonly found to be isozymically polymorphic.

The chemical structure and kinetics of peroxidases in various plants have been studied intensively by many researchers (Theorell 1940, Shannon et al. 1966, Welinder and Mazza 1977), mainly on horseradish peroxidase (HRP). In 1940, Theorell obtained the first crystalline peroxidases from horseradish. Subsequently, molecular weights, isoelectric points and optimum pH were determined (Maehly 1955). It is also known that peroxidase protein contains the heme group as protohaematin IX (Theorell 1940) and is a glycoprotein (Theorell 1942, Darbyshire 1973). Shannon et al. (1966) reported that neutral and amino sugars account for approximately 18% of the weight of peroxidase isozymes in horseradish.

The mechanism of peroxidase reaction was investigated by Chance (1952) and George (1953). In the generally accepted scheme, hydrogen peroxide is combined with the enzyme molecule to form Complex I, then Complex I oxidizes hydrogen. There are several proposed schemes of

formation of Complex II (Santimore 1975). General steps are as follows:

$$\text{Peroxidase} + H_2O_2 \text{ ---------- } \text{Complex I} + H_2O$$

$$\text{Complex I} + AH \text{ ---------- } \text{Complex II} + A + H_2O$$

(AH = any hydrogen donor)

$$\text{Complex II} + AH \text{ ---------- } \text{Peroxidase} + A + H_2O$$

Hydrogen donors act as substrates and form a large and varied group including phenols, aromatic amines (primary, secondary and tertiary), leucodyes and certain heterocyclic compounds, eg. indole (Saunders et al. 1964). The broad substrate specificity of peroxidases implies various physiological functions. Benzidine and o-dianisidine are the most common artificial dyes for peroxidase substrates, and many lignin precursors--ferulic acid, eugenol, guaiacol--and IAA have been observed as peroxidase substrates (Brewbaker and Hasegawa 1975, Halliwell and Ahluwalia 1976). Different isozymes often showed different affinity to these substrates (Srivastava and Van Huystee 1977, Gibson and Liu 1977, Brewbaker and Hasegawa 1975).

2.1.2 Localization and Tissue Specificity.

Tissue polymorphisms of peroxidases are well known in many species (Brewbaker and Hasegawa 1975, Price and Stebbins 1971, Pai et al 1973). Macnicol (1966) found that PxC_3 isozyme of the Alaska pea exists in the roots but not in the leaves. Other isozymes are found mostly in the leaf tissue. It is expected that these tissue-specific peroxidase isozymes would be involved in specific physiological functions. In maize, none of the 13 isozymes were found in all tissues. Px1 and

Px5 were widely dispersed in most tissues, while Px3 and Px7 existed primarily in photosynthetic tissues. Px2 was found only in pollen, and Px9 and Px10 were active mainly in endosperm tissues (Brewbaker and Hasegawa 1975).

The existence of peroxidases in plant cells has been observed histochemically (De Jong 1967, Ridge and Osborne 1970 b). Birecka and Miller (1974) compared the cytoplasmic, ionically bound ($CaCl_2$ extractable) and covalently wall bound peroxidase of several plants including carrot, tobacco and potato. They reported that cytoplasm and cell wall peroxidases differed in their isozyme patterns, activity and reaction to injury. Brewbaker and Hasegawa (1975) reported that in maize, most cathodic isozymes were localized in the cell wall while anodic isozymes were located primarily in soluble fractions. Localization of peroxidases in the cell wall strongly suggests peroxidase involvement in lignification (Birecka and Garraway 1978).

2.1.3 Isolation and Purification.

With the availability of advanced chemical and biochemicl techniques, purification and characterization of peroxidases have been accomplished in various plants including horseradish (Shannon et al. 1966, Welinder and Mazza 1977), tomato (Kokkinakis and Brooks 1979), spinach (Asada and Takahashi 1971), flax (Fields et al. 1977), rice (Ida et al. 1972), pea (Darbyshire 1973), soybean (Flurkey et al. 1978) and wheat (Catedral and Daly 1976).

Shannon et al. (1965) first isolated and purified 7 peroxidase isozymes from horseradish roots. Purification entailed fractionation

of tissue extracts with ammonium sulfate followed by repeated chromatography such as CM- and DEAE- cellulose.

The use of affinity chromatography on Sepharose-bound concanavalin A (Con A) has been reported for purification of peroxidase isozymes (Reimann and Shonbaum 1978). Con A has a general affinity for mannose-containing carbohydrates such as HRP (Reimann and Shonbaum 1978). Brattain et al. (1976) obtained further purification of HRP using Con A with recovery of 73% or more enzyme activity. Darbyshire (1973) separated glycoprotein and non-glycoprotein fractions of pea peroxidases on Con A. Con A was also used for a study of peroxidase synthesis in peanut cells incubated with labelled amino acids. Con A is useful for removing specific groups of molecules from crude extract. Darbyshire (1973) found higher incorporation of radioactivity in the Con A retained fraction. Purification by the Con A method has several advantages over other chromatographic methods for the isolation of many isozymes such as C or HRP. However, certain types of peroxidases such as isozyme A of HRP and the dominant isozyme in sweet potato, interact weakly with Con A (Reimann and Shonbaum 1978). In these cases, the Con A method is not the preferred method for purification.

2.2 Physiological Function of Peroxidases in Plants.

Although the physiological roles of peroxidases in plants have not been clarified completely, it has been suggested that they are involved in several metabolic processes. Peroxidases have relatively broad substrate specificity, intimating diverse roles of peroxidases. The degradation of auxin has also been attributed to peroxidases (Galston

and Dalberg 1954). Catalyzation of the lignin synthesis process has been suggested as another major function of peroxidases (Seigel 1953). Breakdown of phenolic compounds following disease infections, injuries and stress reactions, has been attributed to peroxidase.

2.2.1 IAA Oxidase Activity of Peroxidases.

The nature of enzymes that catalyze the destruction of indole acetic acid (IAA) has been the subject of many investigations. Galston and Dalberg (1954) first proved that peroxidase is involved in the IAA oxidase system of peas. Since then, evidence has accumulated to attribute IAA destruction in plants to peroxidase acting as an IAA oxidase in horseradish (Lee 1977, Endo 1968, Fox et al. 1965, Hinman and Lang 1965, Hoyle 1977), radish (Morita et al. 1962), corn (Lee and Pilet 1977), tomato (Bratton and Henry 1977, Frenkel 1972) and pea (Janssen 1970). Stutz (1957) purified the IAA oxidase-peroxidase from Lupinum albus L. and showed that the enzyme reaction required a phenolic activator, 2,4 dichlorophenol, and was stimulated by Mn^{++}. Hinman and Lang (1965) and Fox et al. (1965) studied the mechanisms of the IAA oxidase enzyme system in horseradish peroxidase (HRP). They proposed that HRP functions as a one-electron oxidizing agent in the peroxidase catalyzed oxidation of IAA. Subsequently, numerous reports indicated that monophenols and m-diphenols also act, in vitro, as cofactors or activators of IAA oxidase (Janssen 1970, Yoneda and Stonier 1967).

The relationship between peroxidase and IAA oxidase, however, is still not precisely understood. Janssen (1970) and Hoyle (1972)

suggested that only one enzyme with two different active centers, is responsible for reactions as peroxidase and IAA oxidase. Srivastava and Van Huystee (1977) reported different Km and optimum pH of four peroxidases in peanut isozymes. They also suggested that only one enzyme functions at different active sites. On the other hand, several papers reported that IAA oxidase and peroxidase are different enzymes. Frenkel (1972) found IAA oxidase activity in ripening fruits independent of peroxidases. Gordon and Henderson (1972) and Lee (1972) could not obtain a direct relationship between IAA oxidase and peroxidase in Avena coleoptile or tobacco tissue cultures. From isozyme studies, Gove and Hoyle (1975) reported that all peroxidase isozymes contain IAA oxidase activity, but many researchers found different activity distributions for the two enzymes. Lee and Pilet (1977) reported that the ratio of activities of the two enzymes varied depending on the location within maize roots. Endo (1968) found that the intensity of the isozyme bands of IAA oxidase and of peroxidase did not correspond to each other. These differing reports indicate that the isozymic relationship of IAA oxidase to peroxidase might vary from species to species. Even though biochemical arguments continue, sufficient evidence has emerged to confirm the role of peroxidase in the destruction of IAA.

2.2.2 Peroxidases in Lignin Biosynthesis.

Polymerization of cinnamyl alcohols is the last step in lignin biosynthesis. Peroxidases have been identified as a main agent in this process (Harkin and Obst 1973, Stafford 1974, Griseback 1977).

Many studies on lignification in plants have revealed a direct relation between peroxidase level and lignification (Siegel 1953, 1957, Stafford 1965, Vance and Sherwood 1976). Using histochemical methods, Harkin and Obst (1973) reported that hydrogen peroxide with peroxidases are necessary for lignin formation in trees. The xylem of trees did not show purple tetramethozyazo-p-methylene-quinone color by application of a precursor syrinaldazine without H_2O_2. They concluded that peroxidase is the only enzyme that polymerizes p-coumaryl alcohol to lignin. Vance and Sherwood (1976) showed lignin formation around infected areas in reed canarygrass leaves with increases in peroxidase activity. When infected tissues were treated by an inhibitor of protein synthesis cycloheximide, no further elevation of peroxidase activity and lignin content occurred.

Involvement of peroxidases in lignin biosynthesis has been further clarified by the identification of peroxidase isozymes in cell walls (Stafford 1974). Histochemical methods for detecting cell wall peroxidase gave information that wall-bound type peroxidases are located in the cell wall where active lignin synthesis occurred (De Jong 1967). Several researchers investigated cell wall peroxidase isozymes quantitatively and qualitatively in horseradish (Liu and Lamport 1974), maize (Birecka and Miller 1974) and tobacco (Mäder 1976). These cell wall peroxidases were demonstrated to be involved in lignin formation. Mäder (1976) reported on the difference in localization and substrate specificity of tobacco peroxidase isozymes G_I, G_{II}, G_{III}. It was shown that <u>in vitro</u> polymerization of coumaryl

alcohol and coniferyl alcohol to lignin-like substances were catalyzed mainly by cell wall peroxidases (Mäder et al. 1977). They concluded that specific peroxidase isozymes at the cell wall would be involved in this process (Vance et al. 1976).

Peroxidase initiating lignin formation requires the presence of hydrogen peroxide. Recently, existence of hydrogen peroxide in cell walls has been reported (Sagisaka 1976). Elstner and Heupel (1976) reported that cell wall-bound peroxidase is responsible for H_2O_2 formation using NADPH or NADH. Gross (1977) found a cell wall-bound malete dehydrogenase in horseradish which provided NADH as an electron donor in the formation of H_2O_2 by cell wall peroxidases.

Regulation of lignin biosynthesis in plant tissues is another interesting subject. In actively growing tissue there is no lignin deposition and plants stop growing when excess lignin is formed. The mechanism of regulation of lignin in vivo, however, has not been clarified. There have been several papers on regulation of lignin formation by IAA (Siegel 1953, 1954, Parish 1969). Siegel (1953, 1954) reported that high IAA levels in elongating tissues suppress peroxidase activity and cause less lignin formation. Rhodes et al. (1976) examined the activity of nine enzymes involved in the biosynthesis of lignin precursor during aging of swede root disks and hydroxylase were found to exhibit 20-30 fold increases in activity while peroxidase showed very little change. Recent studies showed that regulation occurs at the biosynthesis of lignin precursors, rather than during the final polymerization step in which peroxidase is involved

(Grisebach 1977, Gross 1979). They suggested that lignifying tissues, such as xylem, possess the complete set of enzymes required to form lignin precursors compared to tissues such as phloem or parenchyma.

2.2.3 Disease Resistance.

Another possible peroxidase role is related to disease reactions of plants. Qualitative and quantitative changes in peroxidase activity after infection by bacteria, fungi and viruses have been reported in many plants (Farkas and Stahman 1966, Rautella and Payne 1970, Urs and Dunleavy 1974, Vegetti et al. 1975, Veech 1976). The association of peroxidases with disease resistance has also been widely observed. Stahmann et al. (1966) observed an increase in peroxidase activity in a black rot, Ceratocytis fimbriata, Ellisa Halst., on a susceptible variety of sweet potato by exposure to ethylene. Increased resistance was found after the treatment, suggesting that induced resistance is associated with peroxidase activity. Lovrekovich et al. (1968) found that resistance of tobacco to the wild fire disease (Pseudomonas tabaci (Wolf and Foster) F. L. Stevens) was increased by injecting horseradish peroxidase into tobacco leaves. Jennings et al. (1969) compared peroxidase activity in leaf spot, Helminthosporium carbonum Link, in resistant and susceptible maize hybrids. The resistant plants exhibited higher peroxidase activity after infection. In maize, high peroxidase activity was also observed in lines resistant to rust Puccinia sorghi Pers. (Kim et al. 1974), and Helminthosporium maydis Link ex. Fr. (Birecka and Garraway 1978).

The presence of new peroxidase isozymes was observed in various plants after infection accompanied by a rapid increase of peroxidase activity (Lovrekovich et al. 1968, Stahmann and Demorest 1972). Stahmann and Demorest (1972) observed that some isozymes, not observed in healthy tissues or the pathogen, appeared after infection. They suggested several hypothetical mechanisms for peroxidase function in regard to disease resistance, e.g., that peroxidase oxidation products may stimulate synthesis of protein for resistance.

Lignification is a common response to infection (Griseback 1977). Ride (1975) reported that lignification around wounds in wheat leaves was stimulated by inoculation with two non-pathogenic fungi, Botrytis cinerea Pers. ex.Fr. and Mycoshaerella pinodes (Berk & Box.) Stone, but not wounding alone. After the lignin synthesis, attack by pathogens such as Septoria nodorum Berk. and Rob.ex.Desm. was delayed.

Recently, Vance et al. (1976) reported on the relationship of lignin formation in reed canarygrass to disease resistance. They found high lignin formation and high enzyme activity, including peroxidases, in the tissue inoculated with fungus (Helminthosporium avenae Link. The increase of activities was strongly inhibited by cycloheximide, indicating that de novo synthesis of peroxidase was inhibited by inoculation. After the inhibition by cycloheximide, the tissue became susceptible to fungus penetration. Thus peroxidases appear to be a key to unravel the disease resistance mechanism in plants.

2.2.4 Ontogeny.

Variation of peroxidases at different developmental stages has been reported in many species (Alvarez 1968, Gordon 1971, Rychter and Lewak 1971, Dendsay and Sacker 1978). During germination and seedling development, increased peroxidase activity was observed with rapid changes of plant metabolism (Gibson and Liu 1978, McDonald and Khan 1978). Sequi et al. (1970) compared peroxidase isozyme patterns before and after germination in wheat seed. They observed that in ungerminated seeds most of the activity was found in basic isozymes while after germination acidic peroxidase activity increased. Changes in peroxidase activity were also found in rice before and after germination (Navasero et al. 1976). Brewbaker and Hasegawa (1975) reported on peroxidase isozyme changes in young shoots and roots of maize. During mesocotyl development, between 5 and 7 days after germination, isozymes Px3, Px5, Px6 and Px7 increased. A similar increase was observed in coleoptile and root of juvenile maize. In addition to the increase of the peroxidase isozymes, substrate specificity has changed during early growth of some species. Gibson and Liu (1978) reported that peroxidase isozymes of developing pea seedlings between 6 and 8 days showed a large increase in activity with caffeic acid was one of the natural substrates.

Peroxidase activity in older tissues was found to be higher than younger tissues. Gordon (1971) found that major peroxidase isozymes in eastern cottonwood (Populus deltoides Bartr.) were distributed from 4th to 6th leaves, but absent from the apex to second leaves. He

suggested that appearance and intensification of isozymes seemed to parallel the development of photosynthetic activity. However, there was no direct evidence of involvement of peroxidases in photosynthetic system. Recently, Patra and Mishra (1979) examined peroxidase and polyphenol oxidase activities in leaf development and senescence of 16 species. In rice, peroxidase activity was higher in senescent leaves than in primary leaves. Significant correlation was obtained between chlorophyll content and peroxidase activity, but not between chlorophyll and phenol oxidase.

In maize, higher peroxidase activity was found in matured tissue than in juvenile tissues (Brewbaker and Hasegawa 1975, Hamill 1970). During maturation Px3 in leafy tissues developed multiple bands which were regularly spaced and rapidly migrating. From ontogenetic evidence such as changing isozyme patterns, Brewbaker and Hasegawa (1975) suggested that genetic derepression of several maize enzyme loci occurred when specific peroxidases were required in their probable role of lignification or auxin control.

2.3 Response of Peroxidases to Stress.

2.3.1 Environmental Stress.

Plant peroxidase has often been reported to increase under environmental stress such as water, temperature and salt stress (Stutte and Todd 1969, Gerloff et al. 1967, Sheoran et al. 1979). High peroxidase activity was observed under increasing drought stress in wheat (Stutte and Todd 1969). Qualitative changes of peroxidases,

including appearance and disappearance of specific bands, were also reported. Takaoki (19768) however, could not show a definite relation between peroxidase activity and soil moisture.

Elevation of peroxidase activity by cold hardening was found in alfalfa (Gerloff et al. 1967) and Dianthus (McCown et al. 1969). McCown et al. (1969) also observed changes in peroxidase isozyme patterns in two hardy cultivars of Dianthus during hardening. Omran (1980) however, reported no significant differences in peroxidase level in cucumber (Cucumis sativus L.) seedlings exposed to chilling. Only H_2O_2 decrease and IAA oxidase increase were observed.

Sheoran and Garg (1979) found both qualitative and quantitative changes in peroxidases of germinating mung bean under salt stress. New peroxidase isozymes appeared with increasing salinity. They suggested that the existence and variation of isozymes might increase the biochemical versatility of organisms and reduce the loss of function caused by salinity stress. Stevens et al. (1978) however, found no parallel relation between growth responses and changes in peroxidase activity in 11 cultivars of Brassica including B. oneacea L. and B. napa L. under salt stress condition.

Wind is another environmental factor which is expected to influence peroxidases in plants. It has long been observed that wind stress decreases the growth and yield of many crops (Waister 1972, Skidmore et al. 1975, Fryrear et al. 1973, Jaffe 1980). Damage by wind often occurs as surface tissue injury (MacKerron 1976, Precheur et al. 1978), brown lesion formation (Burns 1979) and reduction in plant

height (Akimoto et al. 1975, Russell and Grace 1978). Davies et al. (1978) examined the wind effect on the behavior of stomata in two distinct ecotypes of Cytisus scoparius (L.) Link. They hypothesized that the occurrence of prostrate type subspecies on exposed sea cliffs and the limitation of erect type plants might be related to the stomatal response to wind.

Wind effects on plant metabolism have also been studied under controlled experimental conditions. Grace and Thompson (1973) reported a wind induced reduction of photosynthetic rate in Festuca arudinacea (Schreb.) Wimm., and Todd et al. (1972) reported a 20-40% increase in respiration in wheat, sorghum and soybean. Even though these anatomical and physiological changes implied enzyme level changes, few reports occur on the effects of wind on enzymes such as peroxidase. The effect of flue-curing treatment on tobacco leaf peroxidases was studied by Weston (1968). He reported that peroxidase actively increased after 2 days of treatment and decreased thereafter. The effect of mechanical perturbation (Jaffe and Biro 1978, Biro et al. 1980) and shaking (Ohiri and Danielson 1979, Neel and Harris 1971) were reported to cause retardation of plant growth. Increased secondary xylem growth was observed in these stress-exposed plants and peroxidase change was also implicated (Biro et al. 1980).

Other environmental and artificial stresses have been found to affect peroxidase activity. Horsman and Wellburn (1977) examined plants exposed to SO_2 and found peroxidase activity in the high SO_2 (0.2 ppm) areas to be 50% higher than control plants. They also

observed that plants grown in high SO_2 areas were more tolerant to additional SO_2 exposure. Karege et al. (1979) reported immediate fluctuation of peroxidase level in spinach (Spinacia oleraces L.) leaves after red and far-red light exposure. In a tobacco (Nicotiana tabacum L.) tissue culture system, Leu et al, (1975) detected four new anodic bands in culture media when cells approached death in the dark. Besford and Deen (1977) checked the relationship of nutrient stress and peroxidase level in conifers and found that peroxidase activity increased 11.5 fold by additional iron supply in a liquid feed. Bratton and Henry (1977) showed that small electrical currents (3-7μA) increased peroxidase and IAA level in selected tomato (Lycopersicon esculentum Mill.) tissues such as leaf and petiole. Siegel et al. (1978) examined the influence of experimental hypogravity (0.25 rpm clinostas) on peroxidase activity and lignin accumulation in dwarf marigold and found peroxidase activity to be three times higher than control.

2.3.2 Wounding and Cut Injury.

Plants become wounded by various causes including mechanical injury, cutting and infection by fungi. Increases in respiration, lignification (Rhodes et al. 1976) and polyphenols (Tanaka et al. 1974) are some plant responses to wounding. Increase in the activities of various enzymes such as phenylalaninelyase (Borchert 1978), cinnamic acid 4-hydroxulase (Tanaka et al. 1974), polyphenol oxidase (Hyodo and Uritani 1967) and invertase (Matsushita and Uritani 1974) was also reported as resonse to wounding.

Peroxidase increase by wounding has been observed by many researchers (Birecka and Miller 1974, Matsuno and Uritani 1972, Haard and Marshall 1976). Shannon et al. (1971) first reported on increase in peroxidase activity in sweetpotato slices (Ipomea batatas L.), while Matsuno and Uritani (1972) also found the increase in sweetpotato slices by both cut injury and black rot infection. They observed that an increase in peroxidase activity was stimulated by ethylene and that a lignin-like substance was formed around the injured surface. Similar results were obtained in carrot and potato slices and tobacco internodes (Birecka and Miller 1974). Tanaka and Uritani (1977) observed polarity of polyphenol formation and of various enzymes in the cut-injured sweet potato tissue and found a similar distribution of peroxidase and polyphenol. Borchet (1978) reported perxoidase increase with suberization of wounded potato tuber tissue. Birecka and Catalfamo (1975) investigated the effect of cut-injury and Helminthosporium maydis infection on peroxidase of young maize leaves. Cathodic peroxidases increased as a result of treatments and contributed to the increase of the soluble fraction of peroxidases.

Peroxidase activity increases upon wounding, although there is disagreement about the mechanism of peroxidase increase. Birecka's group found no specific response of peroxidase isozymes (Birecka and Catalfamo 1975, Birecka and Garraway 1978, Haard and Marshall 1976), while Matsuno and Uritani (1972) observed a new peroxidase isozyme formation at the tissue close to the point of injury. The question arises whether increase in peroxidase activity is a result of de novo synthesis of enzymes or enzyme activation. Experiments using

inhibitors such as Actinomycin D and cycloheximide showed de novo synthesis of peroxidases in potato slices (Birecka and Miller 1974) and infected maize leaves (Birecka and Garraway 1978). In carrot slices and healthy maize leaves, however, they found no response to inhibitors. Thus, there still remain questions regarding the mechanisms of enhancement of peroxidase activity.

2.3.3 External Growth Regulators.

There is evidence that externally applied hormones regulate the activity of some plant isozymes. In dwarf maize mutants, gibberellic acid (GA) decreased peroxidase activity and reversed the dwarf genotype (McCune and Galston 1959). Subsequently, Galston and McCune (1961) found GA and IAA altered peroxidase isozyme patterns in dwarf and tall maize. Induction and repression of peroxidase isozymes by IAA was also reported in tobacco pith (Galston et al. 1968, Leshem and Galston 1971), pelagonium pith (Lavee and Galston 1968), oat (Avena sativa L.) coleoptiles (Stuber and Levings 1969), and mung bean cotyledons (Dennsay and Sacher 1978). Lee (1972) reported that kinetin and 2.4 dichlorophenoxyacetic acid (2.4D) also increased peroxidase activity while cycloheximide, actinomycin D and abscisic acid inhibited formation of fast migrating peroxidases. Furthermore, he found an effect of kinetin on cell localization of peroxidase/IAA oxidase, fast-migrating isoperoxidase induced in response to kinetin was only in the cytoplasmic fraction (Lee 1974). High kinetin lowered the specific activity of peroxidase and IAA oxidase.

The effect of ethylene on peroxidases has been studied more intensively than other growth regulators. Ethylene is well known as an endogenous plant growth regulator which is related to various phenomena of plant growth. Ethylene is produced in almost all plant tissues. Massive ethylene production has been observed under conditions where plants suffer from mechanical injury, disease infection and sub-optimal growing environments such as chilling (Wang and Adams 1980) and moisture stress (Jackson and Campbell 1976) as described above. This type of ethylene is commonly called 'stress ethylene' or 'wound ethylene' (Yang and Pratt 1978). The effect of exogenous ethylene on various enzymes in plant tissues has recently been reviewed (Yang and Pratt 1978). Elevation of peroxidase, phenylalanine ammonia lyase (PAL) and polyphenol oxidase were reported, as were increases in oxygen uptake and elevated level of chlorogenic acid in sweet potato root disks (Imaseki 1970, Gahagan et al. 1968). These researchers indicated that small amounts of ethylene (1-10 ppm) were adequate to stimulate peroxidase activity for a short period.

Ridge and Osborne (1970a) found that ethylene increased peroxidases in all parts of Pisum sativum L. plant exposed at concentrations of 1 ppm or more. They suggested that ethylene regulates the activity of peroxidase at a translational level rather than transcriptional level, through experiments using inhibitors such as actinomycin D and cycloheximide. They also reported that ethylene increased peroxidase activity 2-7 times in specific activity both in cytoplasm and ionic bound fractions, but covalently bound fractions were unaffected (Ridge and Osborne 1970b). Since covalent-bound and

other peroxidases were found to contain different isozymes, the response of each isozyme to ethylene must be different. Shannon et al. (1971) observed a 100-fold increase in peroxidase activity in sweet potato slices exposed to 1 ppm ethylene. They supported the Ridge and Osborne (1970) results of de novo synthesis of peroxidases by experiments using basticidin S as an inhibitor. They also showed the incorporation of ^{14}C-leucine is a single band of peroxidase isozymes in ethylene-exposed tissue, implying specific isozyme response to ethylene.

Yang (1968) reported involvement of peroxidases in ethylene biosynthesis using HRP. On the other hand, Galliard et al. (1968) observed that ethylene evolution in apple disks is stimulated by the peroxidation product of linolenic acid but not by methionine which is the most possible ethylene precursor. Mapson et al. (1970), however, observed that ethylene is formed from methionine and not from linolate using labeled substrates.

Fowler and Morgan (1972) examined the relation between auxin-induced ethylene and peroxidases in cotton. Changes in peroxidase and IAA oxidase were observed after the elevation of internal ethylene level. It was indicated that the peroxidase system in cotton (Grossypium tomentosum Nutt.) is not involved in ethylene biosynthesis or at least not as a rate-limiting factor. Although there remain puzzling conflicts among these reports, most current papers (Adams and Yang 1979, Boller et al. 1979) are doubtful about peroxidase involvement in ethylene biosynthesis.

Effects of exogenous substances such as AMO-1618 (2 Isopropyl-4 dimethylamino-5 methylphenyl-1 piperidinecarboxylate methyl chloride), CCC (2-Chloroethyl-trimethylammonium chloride) were also reported (Flückiger 1977, Halevy 1963). Monselise and Halevy (1962) showed an increase of peroxidase by AMO-1618, acting as antagonist to GA which reduced activity in citrus seedling. Harvey et al. (1975) found stimulation of peroxidase activity in maize seedlings by the herbicide EPTC (S-ethyl dipropylthiocarbanate). Lignin deposition was also found during the first few days after treatment. The effect of EPTC on growth and peroxidase was found to be annulled by herbicide antidote (N,N, dially 12-2-dichloroacetamide) but not by IAA and GA. There is still not enough evidence of mechanism between these external substances and peroxidases.

2.4 Peroxidases in Morphological Mutants

2.4.1 Dwarf Mutants.

Increases in the activity of peroxidases have been reported in many dwarf plants: pea (Birecka and Galston 1970, Müller 1969); tomato (Evans 1968, Soressi et al. 1974); maize (Van Overbeek 1938, Shoemaker and Harris 1975); flax (Fields et al. 1976); barley (Price and Stebbins 1971); sorghum (Schertz et al. 1971); cucumber (Miller and George 1979). Van Overbeek (1935) showed in an early study that in a dwarf plant of maize, nana, more growth substances (IAA) were destroyed than in normal, thus causing the inhibition of growth experienced by the dwarf. Peroxidase activity was reported higher in nana than in normal. Also, the amount of auxin in nana, pigmy and

dwarf, dwarf-2, dwarf-3 and dwarf-7 was reported to be lower than normal by Avena coleoptile bioassay (Van Overbeek 1938). However, studies by Shoemaker and Harris (1975) and Hodgdon and Harris (1967) could not confirm the increase in peroxidase from nana.

Kamerbeek (1956) tested the peroxidase content of dwarf and giant plants of Pisum, Phaseolus, Nicotiana and Zea mays. He reported that peroxidase content of dwarf maize seedlings (mesocotyl, coleoptile, primary leaf) was 1-1.7 times greater than that of normal plants. Evans and Alldridge (1965) studied peroxidases in dwarf mutant tomato (Lycopersicon esculentum Mill.). Peroxidase activities were found 3 times greater in the pith, cortex and leaf of the dwarf compared with normal (Evans and Alldridge 1965, Evans 1968). Electrophoretic studies also indicated tissue polymorphism of peroxidase, but no quantitative electrophoretic differences were reported (Evans and Alldridge 1965). Soressi et al. (1974) also reported on the leaf peroxidases in 69 tomato mutants. Several isozyme bands from extreme dwarf mutants were observed with intensive staining. Müller (1969) found correlation between internode length and peroxidase activity in pea (Pisum sativum L.) mutants. The shorter the internodes, the higher the peroxidase activity observed. High activity in dwarf mutants was explained on the basis that peroxidases increased destruction of IAA.

A definitive study of peroxidases in dwarf sorghum was conducted by Schertz et al. (1971) using dwarf and tall strains on isogenic background. They found a significant difference of stem internodal peroxidase activity at 43 to 57 days but no significant differences

were detected at earlier stages. Schertz and co-workers also reported no difference in electrophoretic patterns between the strains.

The control of peroxidase activity by growth regulators and inhibitors may be a key to solving the physiological role of peroxidase in mutants. GA reversible maize mutant dwarf was used by McCune and Galston (1959) to examine peroxidase regulation by GA, but they observed no peroxidase changes in GA treated dwarfs (which grew to normal plant height). Intensive study by Birecka and Galston (1970) showed that GA treatment elongated internodes and inhibited the rise of peroxidase in dwarf pea, yet they could not find any qualitative difference in peroxidase. The manner in which peroxidase activity increases in these dwarf mutants is still not clear.

2.4.2 Other Mutants; Tumorous Tissues, Aborted Leaves.

In tumorous and/or aborted leaf mutants, cell elongation and differentiation occur abnormally. Lignin formation, chlorosis and crinkled tissues are also observed as common phenomena. Price and Stebbins (1971) reported on the peroxidase activity of mature leaves of calcaroides barley which are occasionally twisted or curled. They revealed that a peroxidase increase was detected at developmental stages when histological differences were detected. The electrophoretic characters of peroxidases, however, were alike in both calcaroides and in normal plants. Mathan and Cole (1964) showed an increase in oxidative enzyme activity, including peroxidases, in leaf-shape tomato mutant Lanceolate (La). Mutant La had a narrow and modified leaf at its early stage of growth. They also found that

increase in peroxidase activity and appearance of morphological characteristics exhibited a dosage effect of the gene (La). Further investigation on this La mutant was conducted by Caruso and Glier (1973) and two additional phenol oxidase isozymes, which are not seen in normal plants, were found. Band intensity of these isozymes increased with increasing dosage of the mutant allele. Since these authors used a benzidine-H_2O_2 staining system which is also used for peroxidase detection, these bands can be interpreted as peroxidases. However, they used materials for which the peroxidase genetics were not clear therefore the regulatory system in this mutant has not been clarified.

Kovacs and Maliga (1973) studied the function of the IAA-polyphenol regulation system in genetically tumorous and normal tobacco plants, and in their tissue cultures. The genetic tumors exhibited a high IAA oxidase activity. These researchers provided evidence that close correlation exists between IAA oxidase activity and the chlorogenic acid content of plant tissues. Their results confirmed Bayer's conclusions in 1967 that genetic tumors have both high IAA oxidase activity and high IAA content.

In maize, Gelinas and Postlethwait (1969) examined the IAA oxidases from normal and Knotted (Kn) mutant plants. Knotted mutants develop the phenotype of tumorous finger-like outgrowths on the leaf blade. It was suggested that in these plants a disturbance in the metabolism of phenolic compounds by peroxidase might result in the observed abnormal growth.- Gelinas' group, that the inhibitor

increases with dosage of the Kn alleles up to 18 days after planting, supports their hypothesis of metabolism disturbance.

2.4.3 Morphological Mutants in Maize.

Coe and Neuffer (1977) reviewed numerous studies on variations of morphological characteristics in vegetative and reproductive structures in maize. Dwarfs, presence of abnormal tissue structures, and retarded leaf development are examples of morphological maize mutants. By intensive genetic study, over 70 loci conditioning clear morphological variations in maize structures have been found.

Reduced plant height is characteristic of dwarf maize, but leaf length and internode length of dwarfs have also been shown to be reduced. Scott and Campbell (1969) found that br2 mutant plants have fewer internodes and shorter internode lengths than normal in all stem areas, while leaf lengths were normal. Stein (1955) studied the rate of leaf initiation in dwarf (d) and brachytic (br2) mutant maize. The rate of new leaf initiation was about one new leaf for every 3.5 days in normal and br2, and 5.2 days for d.

Some dwarf mutants have been observed to exhibit growth responses to gibberellic acid. Continuous supply of GA to six dwarf maize mutants, including d, by Phinney (1956) resulted in the development of normal structure maize plants. Other dwarfs, however, such as D8, br, na, na2 and py exhibited little or no response to GA. Brian et al. (1967) applied 143 gibberellins and allied compounds to mutant maize. Differential responses observed of the mutants suggest that mutant genes function at specific stages in the interconversion

and biosynthesis pathway of GA. Van Overbeek (1935) found that the nonGA responding dwarf, na, had a lower than normal auxin level. He suggested that in na higher rates of destruction of auxin (e.g. by peroxidases) exist and may account for the dwarf morphology.

Abnormal vegetative and structural maize plants, such as Corngrass (Cg), grassy tiller (gt) and Knotted (Kn), are another group of morphological mutants. Kn mutant has tumorous tissue along the veins of leaves, while Cg and gt have many tillers in their developmental stages. Nickerson and Hewitson (1963) reported that the number of knots per lateral vein were smaller in heterozygous +/Kn homozygous plants, and that the number of these knots was reduced by application of GA but increased by IAA and butyric acid. The presence of possible IAA oxidase inhibitor formed in Kn seedlings (Gelinas and Postlethwait 1969) was described in the previous section. Physiological studies of Cg and gt have not been reported.

Aborted leaf mutants such as Ragged (Rg) and slashed (sl) were distinctive for their leaf surface. Mericle (1950) conducted morphological and anatomical studies. Though a different leaf developmental pattern was observed in Rg, roots and stems grow as in normal maize. Leaves of Rg were observed with necrotic parts and plasmolytic shrinkage of certain leaf cells was followed by cytolysis of these cells. It was also observed that the degree of manifestation of the Rg character varied with the stage of leaf development.

2.5 Genetics of Isozymes

2.5.1 Isozyme Loci in Maize.

Maize has been a favored plant material for isozyme study due to its genetic variability and accumulated genetic and cytological information. Genetically controlled isozymes have been reported for over 30 loci in maize. Their properties and physiological implications were reviewed by Nelson and Burr (1973) and Coe and Neuffer (1977).

Most isozymes found in maize are under simple monogenic control. Co-dominance, in which heterozygotes are composed of both variant bands of parents, was found in peroxidases Px3 and Px7 (Brewbaker and Hasegawa 1974), esterases, E9 and E10 (MacDonald and Brewbaker 1974) and amylases, Amy and Amy-2 (Coe and Neuffer 1977). At a few loci such as E2, E6, E7, Px5 and Px6, presence vs absence variants were noted. Presence allele Px6-1 was active and intermediate in phenotype in heterozygotes will allele Px6-null (Brewbaker and Hasegawa 1974). Enzymes formed by null alleles were shown to be inactive molecules in Adh (Schwartz 1971).

Several enzymes in maize were reported as dimeric in nature. Schwartz (1960) found an intermediate band as well as both parental bands in heterozygotes of the E locus. Ta (MacDonald and Brewbaker 1972), Adh (Schwartz and Endo 1966) and E3 (Schwartz 1964) were also found to have dimeric nature. Genetic evidence of tetrameric structure has also been found in catalase controlled by Cat (Beckman et al. 1964). In the F1, 3 hybrid bands were observed in addition to the parental bands. Existence of dimeric and tetramic structure was

confirmed chemically by the formation of hybrid bands in vitro following dissociation and reassociation of subunits of catalase (Beckman et al. 1964, Scandalios 1965).

Gene location of isozymes on chromosomes has recently been determined for many loci (McMillin and Scandalios 1981). Esterase: E3, E4 and catechol oxidase Cx were located at chromosome 3, 35 and 10--near du respectively (Kleese and Phillips 1972, Pryor and Schwartz 1973). Roupakias et al. (1980) mapped the catelase genes Cat1, Cat2 and Cat3 using B-A translocation materials. Cat1 was found on the short arm of chromosome 5, 9.1 map units from brittle endospern (bt). Cat2 was on 1S, while Cat3 was located on 1L. They discussed that these recently located catalase loci will enhance further study on the mechanism of catalase regulation in the developing plant. Mapping of biochemical gene markers on the chromosomes will also be necessary for other unmapped loci for further genetic study in maize.

2.5.2 Inheritance Pattern of Peroxidase.

The study of inheritance patterns of peroxidases has been conducted in various plants, with emphasis on the physiological role of peroxidases. Each peroxidase isozyme is generally under the control of only one gene (Benito et al. 1980). Hamill and Brewbaker (1969) first reported genetic control of peroxidascs in maize. The cathodal isozymes were analyzed by segregation pattern of two bands and this locus was designated as Px1. Two isozymes were conditioned by co-dominant alleles as Px1 locus. Work by Hamill (1970), Brewbaker and

Hamill (1972) and Brewbaker and Hasegawa (1974) resulted in the identification of 9 maize peroxidase loci.

In cultivated oats, Smith (1970) found peroxidase polymorphism among 29 winter and 33 spring cultivars of Avena sativa L. and A. byzantina L. Four regions with 12 bands were obtained from the leaves. Two peroxidase bands were conditioned by genes at two loci, one with two alleles and the other with three (Smith 1970). Recently, further study on peroxidase isozymes in oats, including the two isozyme loci designated by Smith (1970), were conducted using diploid, tetraploid and hexaploid wild species and mutants (Yen and Sadanaga 1977). Two independent loci, Px1 and Px2 with 3 and 2 alleles respectively, were detected in leaf blade (Shahi et al. 1969, Pai et al. 1973). From the analysis of F1 and F2 plants, the existence of hybrid bands was found in all combinations of alleles at Px1. They concluded that these isozymes of Px1 were dimers. Furthermore, the existence of a 'regulatory gene' R^{4C} which represses the 4C band coded by $Px2^{4C}$, was found (Pai et al. 1973). Observations of the selfed progenies and hybrids suggested that R^{4C} acted in the leaf blade, with R_{LB}^{4C} acting in the leaf blade and R_{LS}^{4C} acting in leaf sheath. These independently dominant genes resulted in the organ specific intensity variation of the 4C band.

The genetic control of four cathodal peroxidase isozymes in barley was reported by Felder (1976). Two isozymes isolated from root and leaf tissues were found to be independently controlled by two pairs of codominant allelic genes. Felder (1976) indicated that one of the

root isozymes had the same mobility as one of leaf isozymes, but they were under separate genetic control.

Multiple alleles were observed in tomato (Rick and Fobes 1976) and maize (Brewbaker and Hasegawa 1974). In maize, the multiple alleles occurred at 3 loci: Px1 (3 alleles + null), Px4 (3 alleles) and Px3 (6 alleles including double bands). Inheritance patterns of these alleles are codominant except for absence-presence pattern of Px1-null allele. On the other hand, 19 alleles of Prx-4 were found in tomato species (Rick and Fobes 1976). Extremely low recombination rates and the observed concomitant variation of progeny tests showed that all 19 allelic bands were controlled by a single locus, Prx-4. The complex behavior of Prx-4 is unique in both peroxidase and other isozyme inheritance patterns.

Some linkage studies have been conducted of peroxidase isozyme loci. Kobrehel and Feillet (1975) associated peroxidase isozymes a, c, d, with chromosomes 7D, 4B and 7A, respectively in Triticum aestivum. However, they did not locate these peroxidase genes with chromosomal map distances. Hoess et al. (1974) tried to determine the location of a peroxidase locus Px1 in Nicotiana species N. sanderae Hort. ex W. Wats. and N. langsdorffii Weinm. The gene location of Px1 was shown to be between E and R (flower color genes) by the recombination percentages in F_2 plant segregations. Benito et al. (1980) described the inheritance pattern of wheat kernel peroxidases as monogenic inheritance control of eight loci. Two sets of two loci, controlling endosperm peroxidase isozymes, were found to be linked on chromosome

3B and 3D, respectively. In maize, Hamill (1970) studied the linkage of Px3, Px4 and Px5. The study found no evidence of linkage between these three loci.

2.5.3 Maize Peroxidases.

In maize, multiple peroxidases were first reported by McCune (1961). Intensive genetic study of peroxidase isozymes in maize has been conducted by Brewbaker's group since the middle 1960's (Hamill and Brewbaker 1969, Brewbaker and Hasegawa 1974, 1975). At present, thirteen distinct peroxidases have been identified by gel electrophoresis (three cathodal and ten anodal isozymes). Nine of them were reported to be controlled by nine independent loci with 26 alleles (Brewbaker and Hasegawa 1974). These peroxidases are listed below:

Locus	Alleles
Px1	1, 2, 3, null
Px2	1, 2
Px3	1, 2, 3, 4, 5, 6
Px4	1, 2, 3
Px5	1, null
Px6	1, null
Px7	1, 2, null
Px8	1, 2
Px9	1, null

Allelic variants of these loci were reported to include co-dominant or null variants, and no hybrid bands. Genetically marked stocks are

currently available for further studies relating biochemical and developmental functions of isozymes.

Tissue polymorphisms were determined for 13 peroxidases of maize in 21 major tissues or organs (Brewbaker and Hasegawa 1974). Peroxidase isozymes were reported to have high tissue specificity. For example, Px2 existed only in pollen, and Px3 was mainly localized in photosynthetic tissues. Thus selective repression of some peroxidases occurred in many tissues. Ontogenetic polymorphism has also been found for most peroxidases in maize (Brewbaker and Hasegawa 1975) as in other plants (Haard 1973, Birecka and Galston 1970). Brewbaker and Hasegawa (1975) obtained quantitative densitometrical changes of peroxidase zymograms in mesocotyl tissues of maize seedlings.

2.5.4 Alleles of Px3 Locus of Maize.

The genetics of Px3 have been extensively studied in Hawaii by Dr. J. L. Brewbaker, his graduate students and other researchers. Inheritance patterns and gene frequencies have been studied in many inbred lines and tropical races of maize by the Brewbaker group. Px3-2 (Fast) and Px3-1 (Slow) were the two most common bands of Px3 found in these materials. Progeny tests showed clear evidence of monogenic inheritance of the Px3 locus with two alleles: Px3-1 and Px3-2 (Appendix 1).

In 115 inbred lines examined, 60 lines showed Px3-1 while 37 had Px3-2 allele (Appendix 2). In 18 lines, they were segregated. Many field corn lines had Px3-1(S), and more sweet corn lines showed Px3-2(F) except for AA series from the University of Hawaii. These

alleles also segregated in 42 out of 47 tropical races (including teosinte, Zea luxurians examined (Appendix 3). The frequencies of these two bands were obtained from the total number of plants as Px3-2=53.3%, and Px3-1=46.7% (Hardy-Weinberg fitness P = .70 -.80).

Extensive study of Px3 in tropical races of corn maize yielded several new electrophoretic bands which were distinct from the originally located bands. Px3-3 (Super Slow : S*) band was found in Clavo C, a tropical race (Brewbaker, unpublished, Appendix 4b). S* showed slightly slower mobility than S band. Progeny test showed the monogenic inheritance pattern of this allele Px3-3 (Appendix 4a). Gene frequency of S* in Clavo C was calculated as 7.4% and was confirmed to fit the Hardy-Weinberg law in this race.

The Colombian race Puya had additional new bands of Px3. In two races, Puya 2 and Puya 13, an additional band was located in a slightly more anodal region on electrophoresis and was designated Super Fast (F*) (Brewbaker, unpublished, Appendix 5b). By progeny tests and gene frequency (4.4%) the F* band was confirmed as Px3-4 allele (Appendix 5a).

Other Puya races, Puya 10 and 11, contained 7 distinct Px3 genotypes (Appendix 6). A new band, Fast Super Star (F**), was observed in 4 genotypes. F** had higher mobility in electrophoresis than all other bands and was easily distinguished. Also unusual triple band heterozygotes (S, F, and F**) bands were found in these races.

Progeny tests of plants with F** were conducted to prove that F** is a new Px3 allele (Appendix 7). All progenies of F** parents had F** bands. Backcross and selfing of F/F* and S/F** plants also

produced only progenies with phenotypes expected. Genotypes F/F, S/F**, F/F** and F** are shown in Appendix 8. The segregation ratios of these crosses were a good fit with the monogenic inheritance pattern and F** was identified as Px3-5 allele. Gene frequency of Px3-5 (F**) in Puya races was calculated as 11.05% from genotype frequencies of F**, SF**, FF** and SFF**. F** band was not found in any other races and inbred lines studied. Since F** band can be easily distinguished from other alleles, it is useful as a marker gene for genetic crosses.

III. MATERIALS AND METHODS

3.1 Plant Materials

Tropical flint inbred Hi27 and its monogenic mutant lines were used as plant materials. Hi27 is based on Indian inbred CM104 widely adapted to tropics and is highly pest tolerant. Its origin is from the Colombian line, Amarillo Theobromina (Brewbaker 1974). Px3 allelic mutant lines and Px7 allelic mutant lines were also used for characterization of these alleles. A detailed line list is presented in a later section.

Plant materials were grown at the Waimanalo Research Station on Oahu, Hawaii, at 19°N latitude. Greenhouse experiments were conducted either at Waimanalo or Magoon UH upper campus. Plants in the greenhouse were grown in 6" pots.

Seeds were germinated by the 'rolled towel' technique for genotypic studies. Seeds were placed on moist paper towels, which were rolled and stored in beakers in the laboratory at room temperature (25°C). Coleoptiles and leaves of 7-10 day seedlings were used as materials for genotype checks.

All plant materials were kept on ice (0°C) during transfer from the field to the laboratory. For genetic analysis, plant materials were kept in the freezer (-10°C) until use.

3.2 Extraction of Enzyme

For electrophoretic study of phenotypes, samples of tissues such as leaf and coleoptile were macerated in 0.2M phosphate buffer at

pH 7.0. The enzyme solution was absorbed into paper wicks through lens paper barriers to remove fibrous residue of tissues.

For quantitative peroxidase assay, leaves were macerated in a mortar with 10 times their weight of 0.2M phosphate buffer pH 7.0. The homogenate was filtered through a triple layer of cheese cloth, then centrifuged at 10,000g for 20 min. The resulting supernatant was used for peroxidase assay.

3.3 Electrophoresis

Electrophoresis was conducted on horizontal acrylamide gel following the modified method of Brewbaker et al. (1968).

After staining the gel with benzidine-H_2O_2 or O-dianisidine-H_2O_2 the gel was rinsed with running water. The gel was then cut into individual strips and was transferred to a Model 542 Dencicord to determine the isozyme bands using a 0.1 X 6.0 mm aperture and 465 nm filter. The peak areas were recorded on the chart.

3.4 Isolation and Partial Purification of Peroxidases

Crude enzyme solutions were prepared as described in 3.2 except for several changes described below. Leaves (10-20 g) were finely cut and macerated with liquid nitrogen, then homogenized in a Waring Blender in a 0.2M phosphate buffer (pH 7.0).

3.4.1 Ammonium Sulfate Precipitation.

Ammonium sulfate precipitation of crude enzyme was conducted as the first step of purification. Solid $(NH_4)_2SO_4$ was added to crude enzyme solution to 30% saturation. The fraction of under 30%

$(NH_4)_2SO_4$ was collected by centrifuge at 12,000g for 20 min. from the precipitate. Fractions of 30-80% and over 80% were collected from precipitate and supernatant of 80% saturated $(NH_4)_2SO_4$ solutions, respectively. These fractions were dissolved in 5ml of buffer and dialyzed overnight against the appropriate buffer for the following chromatography. The fraction of over 80% was directly dialyzed. The inactive precipitate was removed by centrifugation (12,000g). The 30-80% fraction was used for further purification study.

3.4.2 Affinity Chromatography.

Concanavalin A (Con A) sepharose (Sigma Chemical Co.) was equilibrated with a 0.1M acetate buffer, pH 6.0, containing 1mM $CaCl_2$, $MnCl_2$, and 1M NaCl, in the column (0.7 X 11.5cm) as described by Brattain et al. (1976). The material which was bound to Con A was eluted by 0.11M Mannose in a 0.1M acetate buffer at pH 6.0.

Dye-matrix chromatography was conducted using an Amicon Dye ligand kit including BlueA, BlueB, OrangeA, GreenA, RedA and control (Amicon Co.). Methods followed those of the Amicon technical manual (Pub. I-190, 1980). Each column was equilibrated by 20mM Tris-HCl, pH 7.5, and the sample was allowed to equilibrate for 30 min. The fraction which was bound to the dye-matrix was washed with 20mM Tris-HCl, pH 7.5, and was then eluted by 1.5M KCl in 20mM Tris-HCl, pH 7.5.

Blue sepharose CL-6B (Sigma Chemical Co.) was chosen following screening for efficiency of the dye-matrix. All methods were the

same as those of dye-matrix chromatography except packed in a column (0.7 X 11.5cm).

3.5 Peroxidase Assay

The assay of peroxidase activity was conducted at 25°C by spectrophotometric method using a Unicam SP 1800 Ultraviolet Spectrophotometer.

All peroxidase assays were conducted using O-dianisidine as substrate except in the substrate specificity experiment. The unit peroxidase activity was expressed as an increase in absorbancy per min. (A/min). Total enzyme activity and specific activity were expressed as A/min/g fresh wt. and A/min/mg protein, respectively.

O-dianisidine Assay: Activity of peroxidases was determined by measuring rate of increase in absorbance at 460nm. Reaction mixture contained 1.0 X 10^{-3}% O-dianisidine and 9 X 10^{-4}M H_2O_2 in 0.01M sodium phosphate buffer, pH 6.0 in 3.0ml final volume. The reaction was started with the addition of 100µl enzyme.

Ferulic Acid Assay: Peroxidase activity was measured by increase in absorbance at 400nm. The reaction mixture consisted of 0.01M phosphate buffer pH 8.0, 9 X 10^{-4}M H_2O_2 and 1.667 X 10^{-3}M ferulic acid in 3.0ml final volume. The reaction was initiated with the addition of 100µl enzyme (Gibson and Liu 1978).

Caffeic Acid Assay: Peroxidase activity was measured by the increase in absorbance at 400nm. The reaction mixture contained 0.01M phosphate buffer pH 6.0, 9 X 10^{-4}M H_2O_2 and 1.667 X 10^{-3} M caffeic acid in 3.0ml

final volume. The reaction was started with the addition of 100µl enzyme solution (Gibson and Liu 1978).

Guaiacol Assay: Peroxidase activity was measured by the increase in absorbance at 470nm, with 0.01M phosphate buffer pH 6.0.

Protein was assayed by the Lowry method (Lowry et al. 1951) using bovine serum albumin (BSA) as standard.

3.6 Mutant Study

All the morphological maize mutants used for this study were relatively isogenic with check inbred Hi27. All the recessive mutants were used as homozygotes, while dominant mutants Rg, Cg, Kn were used in the heterozygous condition.

Materials for the mutant study were planted at Waimanalo Research Station during Spring 1979, Fall and Spring 1980. Three plants were randomly chosen and the third leaf down from the youngest initiated leaf was sampled for peroxidase analaysis. The middle section of the leaf, excluding the midrib, was utilized as the experimental material.

Plant height was the measured length from the ground to the extended tip of the longest leaf. Leaf length of mature plants was obtained from the 7th leaf from the top. The 7th leaf was reported to have the highest correlation to total leaf area (Francis et al. 1969).

3.7 Greenhouse Experiment for Wind Treatment

The wind treatment experiment was conducted in the greenhouse at Waimanalo Research Station. Twenty seeds of Hi27, presoaked in water 24 hrs at 30°C, were sown in pots (15cm diameter by 15cm deep)

with a growing medium composed of peat: vermiculite: soil = 2: 1: 1. To prevent rodent damage, pots were covered with wire net until seedling emergence. Ten days after planting, seedlings were thinned to 8 similarly sized plants per pot. Pots in the treatment and control areas were watered by a mist irrigation system which maintained a constant soil water content. A liquid NPK 10-8-7 fertilizer was applied through the irrigation system twice a week. The temperature of the greenhouse ranged between 20°C and 35°C during the experimental period. The difference of temperature between treated area and control was within 2°C.

The wind treatment was conducted as a completely randomized design as described in Figure 1. Two benches were used for the wind treatment area using electric fans (Toshiba 16" oscillating fan). The mean velocity of the wind created from the electric fans was measured with a wind meter (ATR meter W 131, Weather Measure Co.) (Appendix 9). The treatment velocities at the top of plants were 1.8, 3.2 and 4.2 m/s, respectively. As a control, a group of pots were placed on a bench without wind. Within 10 days after planting, when seedlings were approximately 12cm high, treatments were initiated. All pots were rotated every day to allow equal exposure to the wind.

Six plants per treatment were harvested at 0, 3, 7 and 14 days after treatments started for the first experiment and 12 plants after 0, 1, 3, 7 and 14 days for the second experiment. Plant height, fresh weight, dry weight of the whole plants and peroxidase activity of the fully expanded leaves were measured.

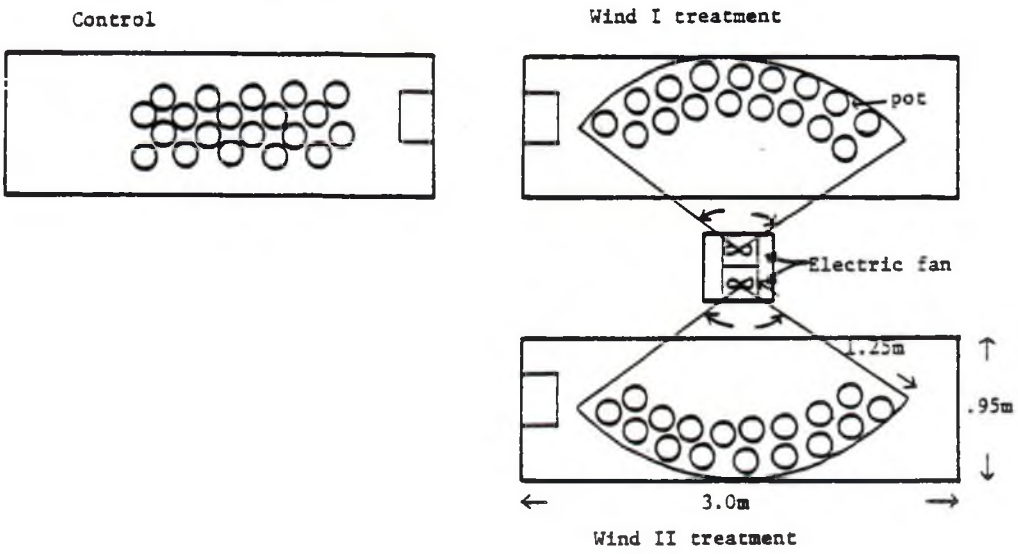


Figure 1. Wind experiment set-up in the greenhouse.

IV. RESULTS AND DISCUSSION

4.1 Genetics of Peroxidases.

4.1.1 Px3 Locus: Inheritance Pattern of Px3-6 Allele ($\overline{SF}$ Phenotype).

Px3 is the major anodically migrating peroxidase isozyme in photosynthetically active tissues of maize such as leaf, coleoptile, mesocotyl, pericarp and brace root. It accounts for about half of the total peroxidase activity in these tissues (Brewbaker and Hasegawa 1975). Px3 is anodically migrating in a 7% acrylamide gel. The Px3 activity in roots is very low and is absent in callus tissues and reproductive tissues, such as pollen and anthers.

The first intimation of an unusual allele at Px3 locus governing the presence of 2 bands was obtained in plants of the race Puya (Appendix 6). These plants carried what appeared to be 3 bands S, F and F**. Since these triple bands (S/F/F**) occurred in a diploid plant, further investigations were initiated.

Progeny tests were conducted to examine whether these double bands ($\overline{SF}$) were under control of a single allele. Plants of SFF** were selfed and F**, SFF** and SF phenotype progenies were obtained (Table 1). Continuous selfing of $\overline{SF}$ band plants, originally produced by SFF** selfing, produced homozygous lines with $\overline{SF}$ double bands in all offspring. Data of Table 1 suggest that the SF double bands ($\overline{SF}$) are controlled by an allele or pseudo allelic complex on the same chromosome. This allele, which produced $\overline{SF}$ bands, was designated as Px3-6.

Table 1. Progeny tests of Px3-6 ($\overline{SF}$) allele.

	Plants with Phenotype					
	F**/F**	SF/F**	S/F	S/S	F/F	
F**/$\overline{SF}$ self	6	38	8	0	0	X^2 = 11.93*
F**/F** X $\overline{SF}$/$\overline{SF}$	0	40	0	0	0	1:1 X^2 = 4.92
$\overline{SF}$/F self	0	0	34	0	18	3:1[1] X^2 = 2.56
$\overline{SF}$/$\overline{SF}$ self	0	0	120	0	0	P = .1-.2

1) S/F:$\overline{SF}$/F:F/F = 1:2:1

Existence of two independent bands of S and F provides evidence that two proteins exist. Thus, at the Px3-6 allele ($\overline{SF}$) at least two cistrons (genes) must exist, on the assumption that one cistron code synthesis of one protein.

The hypothesis which can explain the $\overline{SF}$ allele is that the Px3-6 (F) allele is the result of tandem gene duplication of Px3-1 and Px3-2 alleles (S and F phenotypes) and it, therefore, permits the maintenance of both the S and F bands in a homozygous line. Gene duplication, i.e., two loci arranged in a chromosome in tandem, has been observed in Drosophila (Bahn 1967) and maize (Laughnan 1949, Scandalios 1969). The classic example of tandem gene duplication is Bar locus in Drosophila. The R locus in maize which affects an aleurone and plant anthocyanin colors, and was reported to be a compound locus designated P for plant color and S for seed color (Styles et al. 1973). It is suggested to have evolved from a tandem duplication.

To prove the hypothesis of tandem duplication of S and F alleles, various experiments were conducted. S and F bands were examined to determine whether the bands produced by $\overline{SF}$ allele are electrophoretically identical to those derived from S and F alleles. The progeny test of $\overline{SF}$/F selfing was conducted (Table 1). Segregation of the pheontype was S/F : F/F = 34 : 18 and no other band types were observed. When X^2 analysis was applied using the ratios, $\overline{SF}$/F : $\overline{SF}/\overline{SF}$: F = 1 : 2 : 1 (apparent phenotype SF : F = 3 : 1), the segregation demonstrated a good fit (Table 1). However, X^2 did not fit

the SF : FF = 1 : 1 ratio. Even though phenotype S/F and $\overline{SF}$/F cannot be distinguished on electrophoresis, it is clear that the $\overline{SF}$ allele behaves as other alleles of Px3.

Gene frequency data in the original Puya race was reviewed in light of the equivalence of $\overline{SF}$ bands with S and F bands (Appendix 7). A higher number of phenotypes (S/F) were observed than expected. This was because genotypes $\overline{SF}/\overline{SF}$, $\overline{SF}$/S and $\overline{SF}$/F were electrophoretically similar to the phoenotype S/F. The 83 S/F plants observed were composed of sum of four genotypes--S/F, $\overline{SF}/\overline{SF}$, $\overline{SF}$/F and $\overline{SF}$/S. The gene frequency of Px3-6 ($\overline{SF}$) was calculated to be 26.74% in this race. From this experiment it is concluded that inheritance of $\overline{SF}$ bands is just as that of S and F bands except that both bands are together.

If $\overline{SF}$ bands are identical to those of S and F, then gene dosing will affect the intensity of the Px3 electrophoretic bands. Densitometric tracings of the various genotypes including the $\overline{SF}$ allele were compared. On the gel, a difference in band intensity was observed in F/F, $\overline{SF}/\overline{SF}$ and their hybrid, F/$\overline{SF}$ (Figure 2). Genotype F/$\overline{SF}$ produced a more intense F band than S band. This tendency is clear from densitometric tracing (Figure 3). Two peaks corresponding to S and F bands had the same intensity in $\overline{SF}/\overline{SF}$ and S/F. However, F peak had a higher intensity than S peak in $\overline{SF}$/F. Although the two activity peaks could not be quantitized in exact ratios, the densitometric tracing data of $\overline{SF}$/F suggests a F to S gene dosing ratio of 2 : 1. Dosing effect of F and S genes is additional evidence that S and F of $\overline{SF}$ allele are identical to those of S and F alleles.

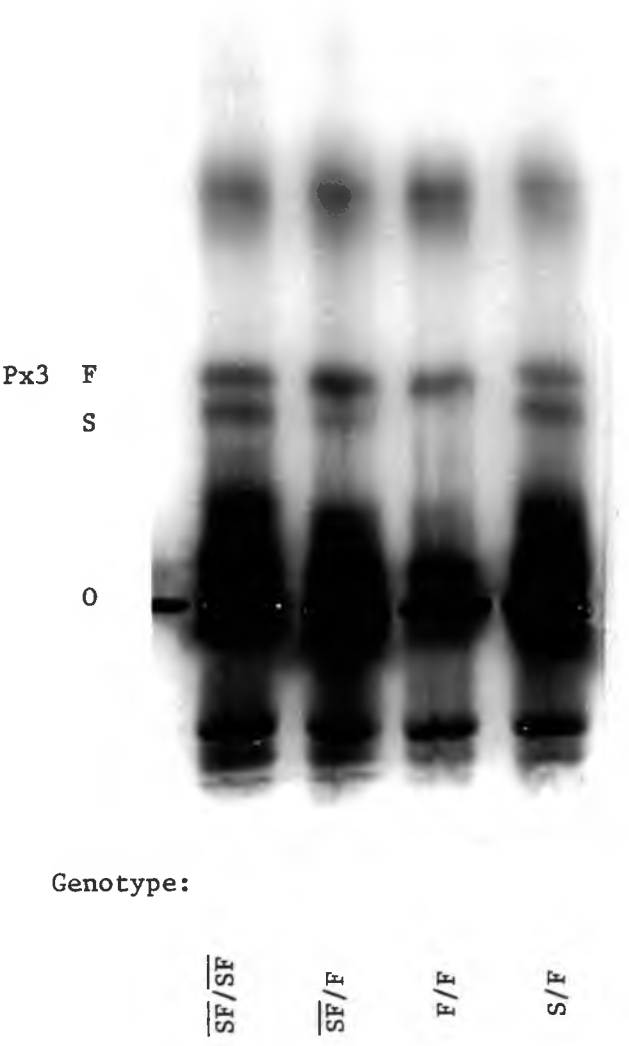


Figure 2. Zymogram of Px3 genotypes, $\overline{SF}/\overline{SF}$, $\overline{SF}/F$, F/F and S/F(check).

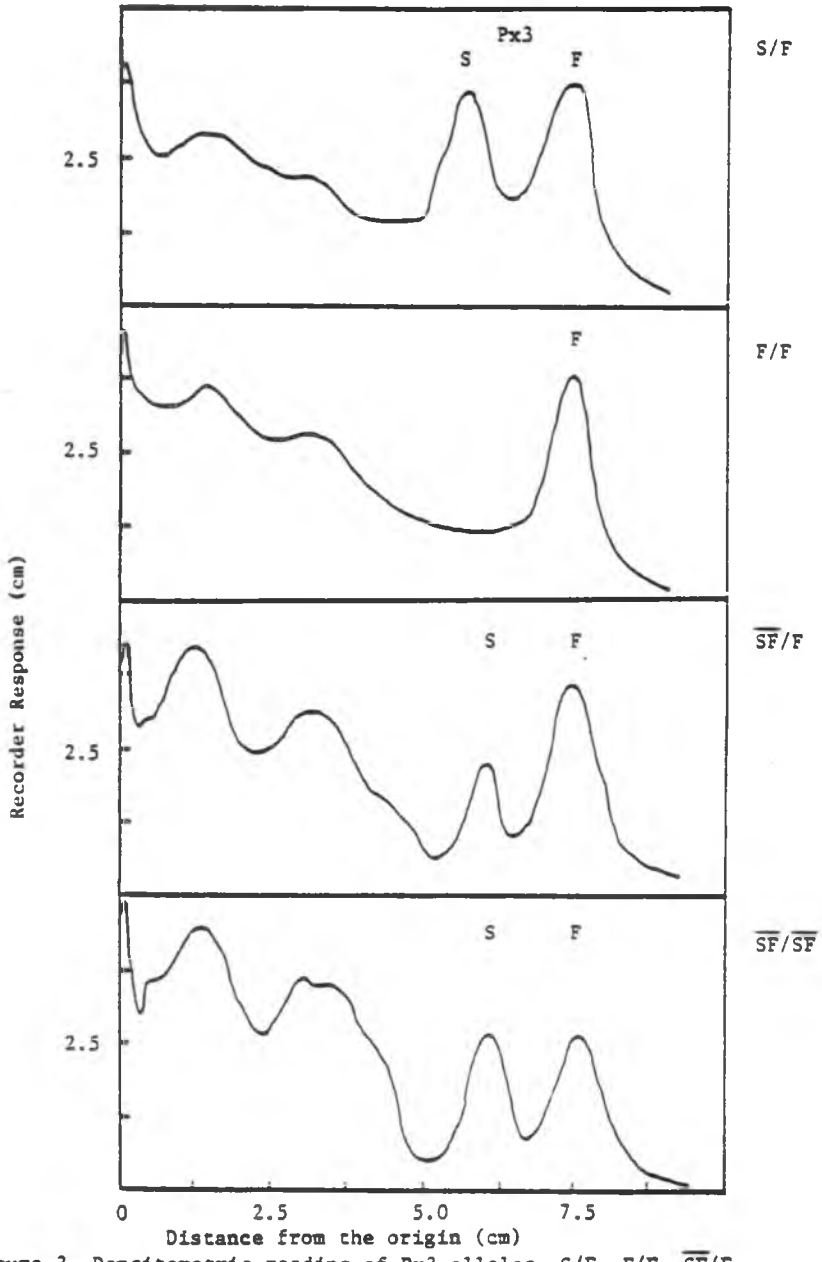


Figure 3. Densitometric reading of Px3 alleles, S/F, F/F, $\overline{SF}$/F, and $\overline{SF}/\overline{SF}$.

To provide additional evidence that S and F bands are identical to $\overline{SF}$ bands and that $\overline{SF}$ allele is the result of gene duplication, the relative mobilities of Px3 alleles at different concentrations of acrylamide gels were examined using Ferguson plots. Ferguson plots are used to detect the contribution of molecular charge and conformation on electrophoretic mobility (Ferguson 1964, Johnson, 1977). The relationship between mobility (Rf) and gel concentration (T) is expressed by the function,

$$\log Rf = \log \frac{Mo}{Uf} + K_R \cdot T$$

$$y \qquad \text{inter-cept} \qquad \text{slope} \quad x$$

where Mo is the theoretical mobility of the molecule, Uf is a buffer constant, K_R is the retardation coefficient and Rf is the observed relative mobility of the molecule. Johnson (1975) explained that variance of K_R between proteins may reflect molecular weight differences resulting from size or subunit aggregation dissimilarities. To compare two isozymes, slope (K_R) corresponds to molecular sizes, while intercept ($\log \frac{Mo}{Uf}$) shows molecular charges. Comparison of these two values obtained from S and F bands of Px3-6 ($\overline{SF}$) and Px3-1 or Px3-2 were used to identify the hypothesized gene duplication.

Leaf samples of genotypes Px3-1 (S), Px3-2 (F), and Px3-6 ($\overline{SF}$) were prepared and electrophoresced using procedures described in Section 3.3. Gele concentrations of 4, 5, 6, 7, and 8% were used. Ferguson plots of F and S bands of Px3 in four Px3-2 (F) lines; 442, B70, CI66 and H60 and six Px3-1 (S) lines; AA8, B77, H55, H95, Hi27,

and Hi31 were also examined. Slopes (K_R) and intercepts ($\log \frac{Mo}{Uf}$) were compared among the inbred lines used and between S and F bands (Table 2). No differences were found among inbred lines in K_R and $\log \frac{Mo}{Uf}$. Log $\frac{Mo}{Uf}$ of S was higher than that of F. Furthermore, slopes (K_R) and intercepts $\log \frac{Mo}{Uf}$ of $\overline{SF}$ bands were compared with those of S and F bands (Table 2). There were no significant differences in slopes or intercepts between $\overline{SF}$ band and S and F bands. These results strongly suggest that S and F bands from Px3-6 (SF) alleles are electrophoretically identical.

Ferguson plots of $\overline{SF}$/F** plants were also examined (Figure 4). Analysis of variance of K_R and $\log \frac{Mo}{Uf}$ values from S, F and F** indicated that only $\log \frac{Mo}{Uf}$ had significant differences among these bands (Table 2). Mobility differences between Px3-1 (S), Px3-2 (F), and Px3-5 (F**) may be due to single amino acid substitutions rather than binding of ions or other low molecular weight compounds. In comparing allelic proteins, amino acid substitutions have been commonly reported. Px3 alleles in maize can be concluded as one of those types of modifications.

Results of these three experiments: 1) inheritance pattern, 2) densitometric scans, 3) Ferguson plots, provide the evidence necessary to accept the hypothesis that $\overline{SF}$ bands are electrophoretically identical to S and F bands of Px3. These results also indicate that the Px3-6 ($\overline{SF}$) allele is a probable tandem duplication of Px3 genes.

Tandem duplication of two electrophoretic loci was observed in several systems (Nelson and Burt 1973). Bahn (1967) found two closely

Table 2. Relative mobility of Px3 allelic bands as a function of acrylamide concentration.

Line	Px3 band	Number of Plants	Slope (K_R) x 10^{-2}	Intercept ($\log \frac{Mo}{uf}$)	
442a	F	2	-3.03 ± 0.13 [1]	-0.359 ± 0.019	
B70	F	2	-3.07 ± 0.07	-0.359 ± 0.017	
CI 66	F	2	-2.78 ± 0.40	-0.364 ± 0.012	
H60	F	2	-2.98 ± 0.08	-0.391 ± 0.013	
mean			-2.94 ± 0.01	-0.368 ± 0.005	b [2]
AA8	S	2	-2.61 ± 0.14	-0.409 ± 0.023	
B77	S	2	-3.26 ± 0.19	-0.445 ± 0.037	
H55	S	2	-3.29 ± 0.47	-0.440 ± 0.024	
H95	S	2	-2.52 ± 0.11	-0.451 ± 0.001	
Hi27	S	2	-4.06 ± 0.34	-0.468 ± 0.011	
Hi31	S	2	-3.44 ± 0.44	-0.440 ± 0.016	
mean			-3.20 ± 0.18	-0.442 ± 0.001	c
SF/F**	F	10	-3.26 ± 0.24	-0.341 ± 0.016	b
	S	10	-3.79 ± 0.18	-0.400 ± 0.010	bc
	F**	10	-3.43 ± 0.15	-0.235 ± 0.009	a

1) S.E.
2) Means followed by the same letter are not significantly different at the 1% level according to Duncan's multiple test.

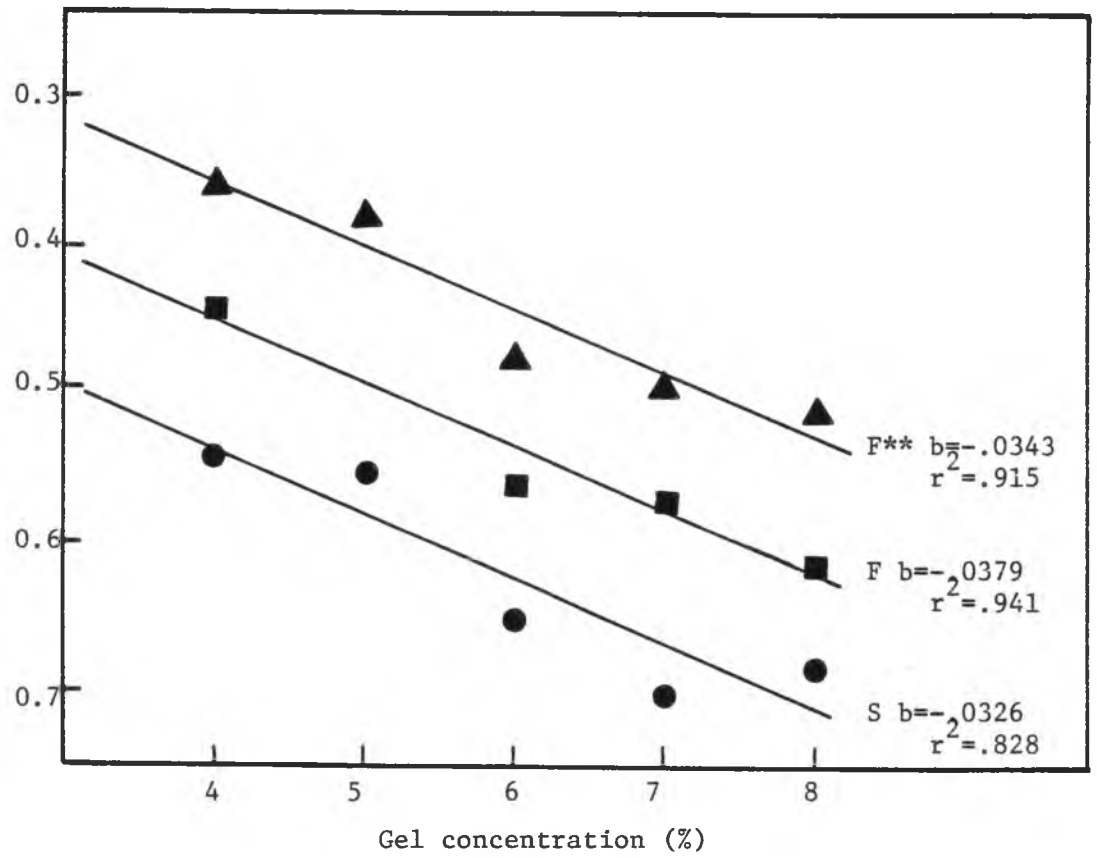


Figure 4. An example of Ferguson plots of SF/F** plant (Row 441-4).

linked amylase loci, Amy-1 and Amy-2 in Drosophila. Offspring (5039 individuals) of the test cross of these alleles contained two recombinant phenotypes of Amy-1 and Amy-2. It was concluded that these two loci resulted from a gene duplication due to unequal crossing over between Amy loci. In maize, closely linked alcohol dehydrogenase loci, Adh1 and Adh2 were found as a gene duplication (Scandalios 1969). In 20,124 kernels of F2 progeny, three recombinants between Adh1 and Adh2 were recovered (Scandalios 1969). In 20,124 kernels of F2 progeny, three recombinants between Adh1 and Adh2 were recovered (Scandalios 1969). Since these experiments require a large sample size, sufficient statistical analysis to confirm recombination within the $\overline{SF}$ phenotype has not been conducted. Lewontin (1970) noted that the possibility of observing the recombinants of tandem duplication gene is extremely low.

Further investigation of gene duplication at $\overline{SF}$ allele of Px3 may be pursued by utilizing a method similar to Bahn (1967) and Scandalios (1969). By this recombination test, it will be clear that if the two genes (cistron) can be separated by crossover or not. Figure 5 shows the test crosses of SF/F** which will produce distinctive recombinant genotypes. If there is crossover between S and F genes of $\overline{SF}$ allele (either scheme a) or b)), SFF** and F (is F/F) or SFF** and (is S/S) plants will be obtained. Frequency of these recombinants, however, will be very low and a large number of progenies will be required. If Px3-6 ($\overline{SF}$) is a result of gene duplication, it is a rare example of electrophoretic allele duplication at a locus.

Genotype of male parent: (SFF**)		Male gametes	Genotype of female parent: (FF or SS) Female gametes F	S
a) S F / F** →	Parental:	SF	SF/F	SF/S
		F**	F/F**	S/F**
	Recombinant:			
		SF**	SF/F**	S/F**
		F	F/F (detectable)	S/F
b) S F / F** →	Parental:	SF	SF/F	S/F
		F**	F/F**	S/F**
	Recombinant:			
		FF**	F/F**	SF/F**
		S	S/F	S/S (detectable)

Figure 5. Possible recombination tests for recombination between $\overline{SF}$ duplication allele of Px3.

4.1.2 Chromosomal location of Px3 locus.

The chromosomal location of Px3 locus was studied using genetic markers on chromosome 7 and Px3 mutant alleles. During a sabbatical leave in Hawaii, Weber (1975, unpublished) showed that Px3 locus is located on chromosome 7, using Trisomic materials. Therefore, four morphological mutant genes on chromosome 7 with distinct phenotypes were chosen as genetic markers to determine the Px3 chromosomal location (Table 3). They are opaque (o_2), slashed leaf (sl, Teopod (Tp), and Papyrescent glumes (Pn). All mutant lines were isogenic to Hi27 by continuous backcrosses (Brewbaker 1974).

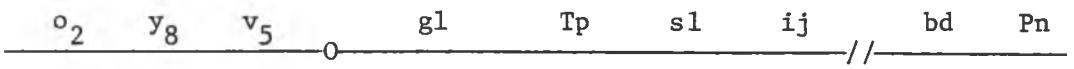
Test crosses for the linkage study were conducted between heterozygous gene marker stocks and homozygous tester stocks (Table 4). At the Px3 locus, Px3-5 (F**) allele was introduced to detect contamination during pollination.

All plants were grown in field conditions (1979 spring, and 1981 spring) to check phenotypes of Px3 with marker genes. Young leaves at two to three week stages were harvested from each plant. Px3 genotypes were determined by duplicated gel zymograms as described in Section 3.3. Phenotypes of genetic markers were checked at later stages of plant growth except for opaque (o_2) phenotype which was checked at pre-planting stage, because its phenotype is expressed in the endosperm.

Four groups of genotypic segregations were obtained in the 4 test crosses from distinctive morphological phenotypes and Px3 electrophoretic mobilities. Chi-squares were calculated on the

Table 3. Phenotypes and location of genetic markers used for Px3 linkage study.

Chromosome 7



Symbol	Genetic Marker	Location	Phenotype
o2	opaque endosperm	7-16	endosperm starch; soft, opaque
Tp	teopod	7-46	many small partially podded ears; narrow leaves
sl	slashed leaf	7-50	leaves slit longitudinally by necrotic streaks
bd	branched silkless	7-109	ears branched at base; without silks; tassel spikelets in groups of two or more
Pn	papyrescent glumes	7-112	long thin papery glumes on ear and tassel

Table 4. Linkage between Px3 alleles S (Px3-1) and F (Px3-2) and o_2, s1, Tp and Pn.

Marker Gene (location)	Cross	Plant Genotype parental		Plant Genotype recombinant		X^2 [1]	P
o_2 (7-16)	$\frac{o_2F}{o_2F} \times \frac{o_2F}{+F^{**}}$	$\frac{o_2F}{o_2F}$	$\frac{o_2F}{+F^{**}}$	$\frac{o_2F}{o_2F^{**}}$	$\frac{o_2F}{+F}$		
	obs.	42	38	50	50		
	total	80		100		2.22	.5-.9
s1 (7-50)	$\frac{s1S}{s1S} \times \frac{s1F}{+F^{**}}$	$\frac{s1S}{s1F}$	$\frac{s1S}{+F^{**}}$	$\frac{s1S}{s1F^{**}}$	$\frac{s1S}{+F}$		
	obs.	28	46	26	33		
	total	74		59		1.69	.1-.5
Tp (7-46)	$\frac{+S}{+S} \times \frac{TpF}{+F^{**}}$	$\frac{TpF}{+S}$	$\frac{+F^{**}}{+S}$	$\frac{TpF^{**}}{+S}$	$\frac{+F}{+S}$		
	obs.	44	42	38	27		
	total	86		64		2.92	.05-.1
Pn (7-112)	$\frac{+S}{+S} \times \frac{PnF}{+F^{**}}$	$\frac{PnF}{+S}$	$\frac{+F^{**}}{+S}$	$\frac{PnF^{**}}{+S}$	$\frac{+F}{+S}$		
	obs.	25	30	6	4		
	total	55		10		31.16**	.01

1) X^2 was calculated on the hypothesis that Px3 and these genes are unlinked, i.e. an expected ratio of 1:1 parental:recombinant.

hypothesis that Px3 and the marker gene were unlinked and would segregate 1 : 1 for the parental and recombinant types.

A significant linkage ($P < .01$) was obtained between the marker Pn and Px3. However, no evidence was found of linkage between Px3 and the three gene markers o_2, sl and Tp (Table 4). Recombination percentage between Px3 and Pn was determined to be 15.38%. Pn is located on the end of chromosome 7, at map distance 112. Therefore, the probable Px3 location is expected to be on the linkage map 96.62 from this linkage test result.

The waxy translocation method (Anderson 1938) was used as another linkage test. Wx7 - 9a line which contains the translocation of chromosome 9 with waxy (wx) gene was used for the test. The break points are at 7L.63 on chromosome 7 and on chromosome 9 is at 9S.07 (Lambert 1979). Since the wx gene is located at 0.3 genetic distance from break point 9S.07 on chromosome 9, the wx phenotype can generally be used as a marker for the translocation point (Pryor and Schwartz 1973). Heterozygous material for Px3 locus (S/F) and translocation material wx /+ was produced by repeated crosses. As a tester, homozygous materials $\frac{wx}{wx}\frac{S}{S}$ were used. The chromosomal configuration of wx7 - 9a translocation is shown in Figure 6. Eight hundred and thirty-nine plants were examined for both wx and Px3 phenotypes. The segregation of parental and recombinant plants is shown in Table 5. A Chi-square analysis showed that the linkage between Px3 and wx (translocation) was significant. The recombination percentage between wx and Px3 loci was calculated to be 41.52 ± 2.50 (Table 5).

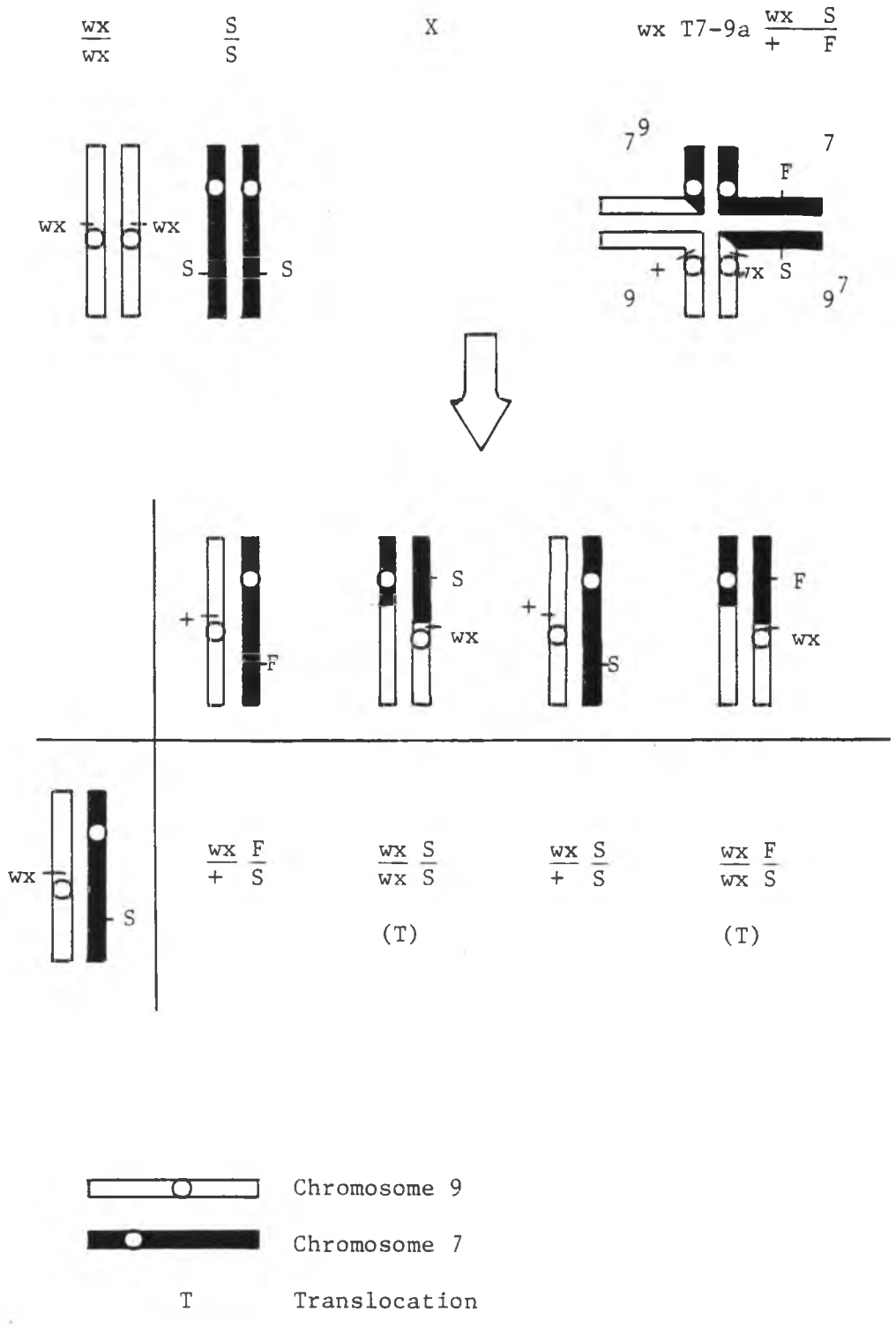


Figure 6. Chromosomal configuration of wx7-9a translocation.

Table 3. Linkage between Px3 and wx at wx 7-9a translocation line.

Cross	$\frac{+}{wx_T}$ 2) $\frac{S}{F}$ X $\frac{wx}{wx}\frac{S}{S}$						
	Plant Genotype						
	Parental		Recombinant		X^2 1)	P	Recombination %
	$\frac{wx_T}{wx}\frac{S}{S}$	$\frac{wx}{+}\frac{S}{F}$	$\frac{wx_T}{wx}\frac{S}{F}$	$\frac{wx}{+}\frac{S}{S}$			
Experiment 1.							
obs.	109	129	104	87			
total	238		191		5.15**	.01	44.50
Experiment 2.							
obs.	139	111	73	87			
total	250		160		24.65**	.01	39.02
							$\bar{X}$ = 41.52+2.50

1) X^2 was calculated on the hypothesis that Px3 and wx are not linked, i.e. expected ratio of 1:1 parental : recombinant.
2) Translocation

Chromosomal location of break point of wx-translocation (7L.63) was calculated by Phillips (1969). The interchange stocks of chromosome 6 and 7 (break point 7L.63) were examined on linkage with 7L genetic markers, such as ij, sl, Bn, and bd. Map unit 6.8 from ij (7.52) was reported as the most probable chromosomal location of 7L.63 break point (Phillips 1969, Beckett et al. 1978). The break point 7L.63 can be mapped as 7 - 58.8 using this information. Since the recombination percentage is 41.52 ± 2.50, and Px3 showed no linkage with genes proximal to ij (Table 4), a possible Px3 location was calculated to be 100.32 ± 2.50. Based on the four independent crosses and the waxy translocation method it is suggested that a Px3 gene location between 96.62 and 100.32 is most probable.

Gene location of cathecholoxidase (Cx) in maize was determined by using similar methods, wx translocation materials and a marker gene du (Pryor and Schwartz 1973). After the general location of Cx was determined based on the wx-translocation method, a chromosome 9 marker gene du closest to the break point was chosen adn the Cx - du linkage was examined. Since there are no mapped genes available between 80 - 100 map units on chromosome 7 in maize, it is difficult to test linkage with the genes which are expected to be closely linked with Px3.

The Px3 gene location identified here should be confirmed by using the three point testcross method. The more generations will be required to test recombination percentages of Px3 and two genetic markers.

Once chromosomal locations are determined, isozyme loci are useful as gene markers on the chromosomes. Kleese and Phillips (1972) reported the use of Esterase locus (E4) for detection of chromosome aberrations in maize. Usually to detect translocated chromosomes, plants must be grown to maturity to check for pollen sterility. Use of isozyme mutants E4 permits identification of fertile and semifertile plants by their E4 genotypes long before maturity. Px3 locus can also serve as a marker for the chromosome 7 long arm region. Px3 produces distinct bands on zymograms, permitting rapid detection in seedlings of electrophoretic genotypes. Moreover, the use of Px3-5 (F**), not found in common inbred lines, can avoid mistakes from contamination.

4.1.3 Px7 locus.

Maize peroxidase isozyme 7 (Px7) is a major source of peroxidase acting in leaf, silk, root and seedling tissue (Brewbaker and Hamill 1972, Brewbaker and Hasegawa 1975). This enzyme stains as a large diffuse region which remains near the origin in standard acrylamide gels. Three alleles, Px7-1 (Slow), Px7-2 (Fast) and Px7-null were found in various inbred lines of maize. In the inbred lines screened, 65 showed Px7-1 (Slow), 3 inbreeds (T24, T36, T55) showed Px7-2 (Fast) and 5 were Px7-null (Liu, Nagai and Brewbaker 1981).

The separation of these alleles was not always clear on standard acrylamide gels. To improve resolution of Px7 bands, modifications of the standard method were examined. Extended electrophoresis, 35-40 hours, in a less dense 6% acrylamide gell system, provided the best

separation of Px7-1 and Px7-2 bands. Use of surfactants such as Triton X-100 and changing the pH gave no improvement of the resolution.

Segregation of Px7 alleles was observed clearly and independently of other peroxidase loci on the 6% long duration gels (Figure 7). Heterozygous plants showed two bands, Slow and Fast, corresponding to each of the parental alleles. No evidence of hybrid bands was obtained in all Px7 heterozygous plants. This is an indication that Px7 is a monomer, as are the other maize peroxidases.

Densitometrical tracings of Px7 bands are shown in Figure 8. Hi27 (Px7-1 S/S) had a peak very close to the origin (Figure 8a), while T55 (Px7-2 F/F) contained a large peak for the F band Figure 8b). Hybrid T55 X Hi31 (S/F) yielded S and F peaks and no middle position peaks (Figure 8c). A null variant CI66 (Px7-null) showed no peaks (Figure 8d). Same results were obtained from Hi27 (Px7-1 S/S), T36 and T24 (Px7-2 F/F) and B37 (Px7-null).

Progeny tests of Px7 alleles were conducted to confirm the Px7 inheritance pattern (Table 6). Hybrids from the cross S/S x F/F; (B68 x T24, Hi27 x T24) showed all S/F phenotypes. Backcross of S/F x F/F and selfing of S/F yielded the expected inheritance patterns, SF : FF = 1 : 1 and SS : SF : FF = 1 : 2 : 1, respectively. These results confirmed the monogenic inheritance pattern of Px7.

CI66 was observed to be null in all the tissues examined, while B37 was absent in coleoptile and silk but present in leaves in previous studies (Brewbaker, unpublished). The inheritance of Px7-null was not clarified by these crosses studied. The F1 of B37

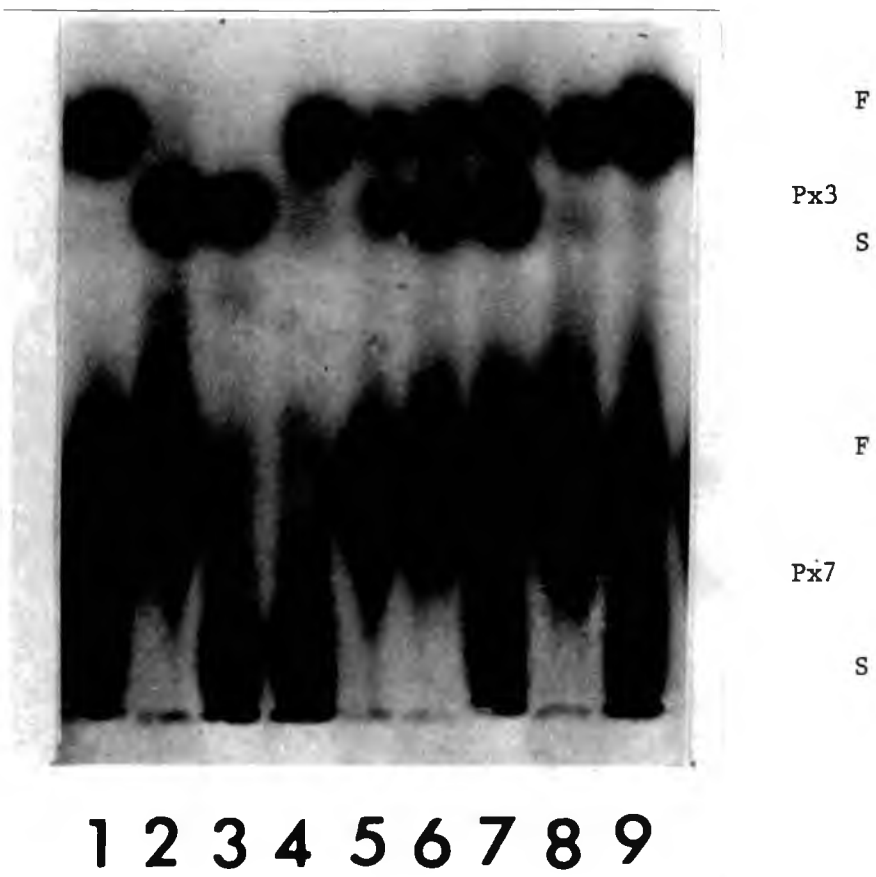


Figure 7. Zymogram showing independent segregation of Px7 and Px3 alleles. Plants 1-9 were from selfing of a plant heterozygous Px3-1/ Px3-2 and Px7-1/Px7-2.

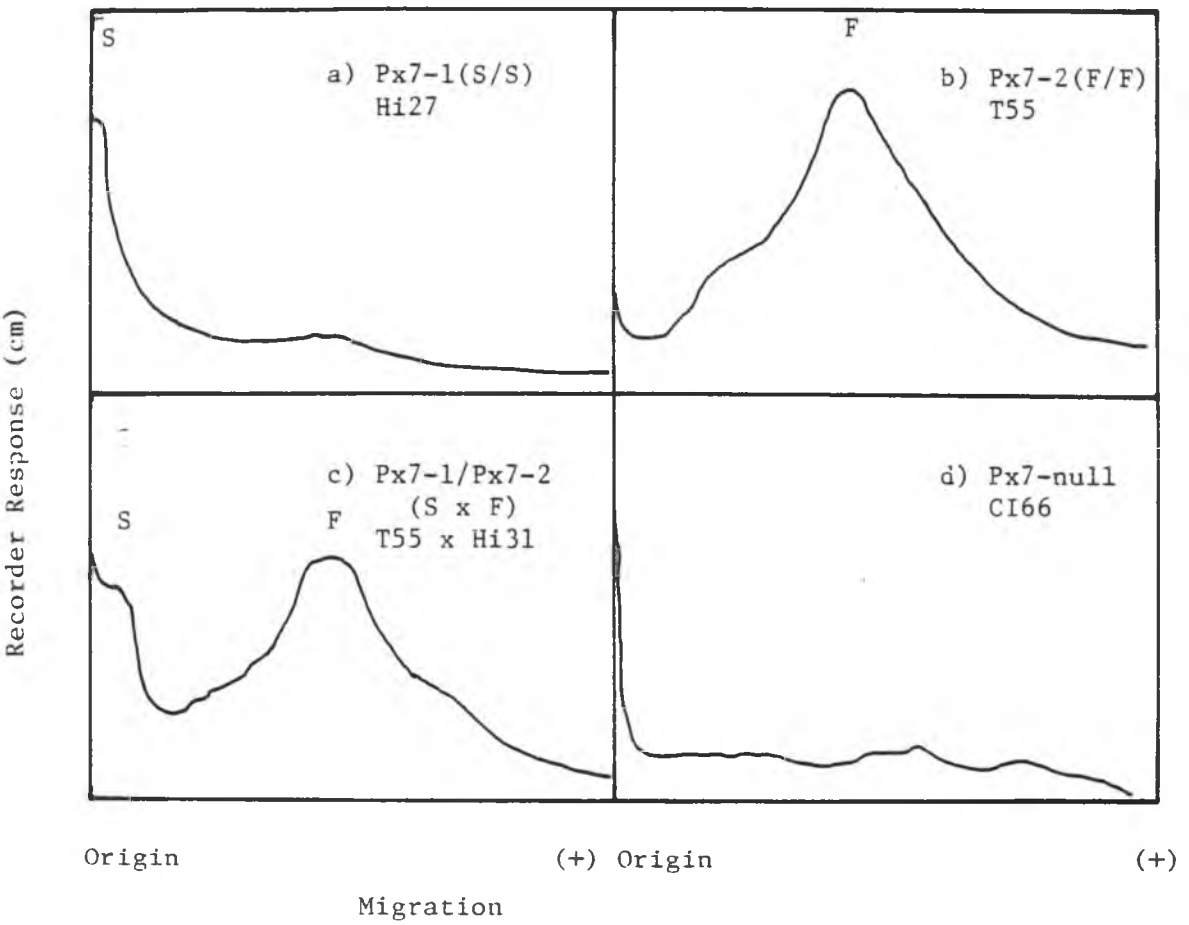


Figure 8. Densitometry of Px7 bands: (a)S/S,(b)F/F, (c)S/F and (d) null.

Table 6. Progeny tests of *Px7* alleles in maize.

Cross	Plants with Phenotype S	S/F	F	X^2	P
Hi27 X T24 (s) (F)	0	50	0	-	-
B86 X T24 (S) (F)	0	18	0	-	-
(T36 X Hi27) X T36 (S/F) (F)	0	14	17	.290	.70-.90
(T55 X Hi27) X T55 (S/F) (F)	0	7	8	.067	.90-.95
(T24 X Hi27) self (S/F)	21	43	17	.703	.50-.70
CI66 X T24 [1] (null) (F)	0	12	0	-	-
B37 X Hi27 [1] (null) (S)	23	0	0	-	-
B37 X T24 [1] (null) (F)	0	3	2	-	-
CI66 X B37 [1] (null) (null)	12	25	0	-	-

1) Coleoptile tissues were used for analysis.

(null) with Hi27 (S) yielded the expected phenotype S in coleoptiles. However, crosses between nulls, CI66 (null) X B37 (null) resulted in S : SF = 12 : 21 which was not expected from this cross. CI66 (null) X T24 (F) showed a S/F phenotype instead of the F which was expected and so did CI66 (null) X Hi27 (S). This poor fit of null alleles inheritance to monogenic Mendelian ratios may be related to two kinds of nulls at Px7 locus. In a "true" null variant of CI66 no Px7 activity was found, thus the protein coded by Px7 might be inactive as a peroxidase. On the other hand, "leaky" nulls may have active Px7 protein, since in leaf tissues they showed Px7 activity. However, in silks, repressor or regulatory genes might suppress Px7 activity. The presence of regulatory genes was reported in rice peroxidases (Pai et al. 1973) and maize alcohol dehydrogenase (Schwartz 1966). In both cases, regulatory genes controlled the appearances of bands which are coded by structural genes.

Further investigations of inheritance patterns and tissue specificity of Px7-null variants will be required to understand the null allele mechanisms including the possibility of Px7 regulatory genes.

4.2 Biochemical Characterization of Peroxidases Px3 and Px7.

Partial purification and some biochemical characterization of two major peroxidase isozymes of maize leaves, Px3 and Px7, were conducted. The genetic and tissue differences of these two isozymes in maize were clarified by other researchers (Hamill 1970, Brewbaker and Hasegawa 1974) and in the previous section. Both isozymes are controlled by independent monogenes. Px3 has 6 alleles without a null variant, while Px7 has 4 possible alleles including two kinds of null variants. Behavior of the two isozymes on electrophoretic gels is different. Px7 remains near the origin, while Px3 migrates 25-30% of the anodal front on zymograms.

A difference in tissue and intracellular localization of the activities of Px3 and Px7 was reported by Brewbaker and Hasegawa (1975). Both enzymes are widely distributed in various maize tissues including leaves and coleoptiles. Only Px7 was found in the silk, however. Px3 was found equally in the soluble and cell-wall fractions, while the cell-wall fraction of Px7 dominated over the soluble fraction (Brewbaker and Hasegawa 1975).

All differences in the properties examined suggest biochemical dissimilarity of the two enzymes. In this section, partial purification of the two isozymes, Px3 and Px7, is reported and Km values of the two isozymes, including allelic enzymes at each locus, are compared.

4.2.1 Partial Purification of Px3 and Px7.

A crude enzyme solution was obtained from 20g mature leaves of the Hi27 inbred line. Ammonium sulfate fractionation was conducted as described in section 3.4 and the 30%-80% saturated fraction was collected for further experiments. In the 30%-80% $(NH_4)_2SO_4$ fraction, 70% of the original activity was recovered. The ammonium sulfate fractionation caused a 5.95 fold increase in specific activity of peroxidase (Table 7).

Affinity chromatography using Concanavalin A-Sepharose 4B (Con A) was the next step of purification. Dialyzed solution (1 ml) from the $(NH_4)_2SO_4$ fraction was applied to a 0.7 x 11 cm column of Con A. The column was washed with 50 ml acetate buffer and was eluted, the acetate buffer containing 0.11 M mannose. Fractions of 1.30 ml were collected.

Figure 9 shows the elution pattern of Hi27 peroxidases. The first peak (Peak I, fractions 4-7) showed high 280 nm absorbance of protein and low peroxidase activity. The washed peroxidases did not bind to Con A. On the other hand, the second peak (Peak II, fraction 30-33), which bound to Con A, had low 280 nm absorbance with high peroxidase activity.

The proportions of the peroxidase activity in the two peaks and the relative specific activities are summarized in Table 8. The combined yield of peroxidase activity in Peak I and Peak II was 72.0% of the original activity added to the Con A. The recovery of the Peak II was 53.0%. The relative specific activity in Peak II was

Table 7. Ammonium sulfate fractionation of maize peroxidases.

Fraction	Total Activity	Total Protein	Specific Activity
	--A/min--	--mg--	--A/min/mg protein--
Crude enzyme	12740	2259.06	5.64
Under 30% $(NH_4)_2SO_4$	205.9	74.43	2.77
30-80% $(NH_4)_2SO_4$	8857.8	264.00	33.55
Over 80% $(NH_4)_2SO_4$	601.5	180.45	3.33

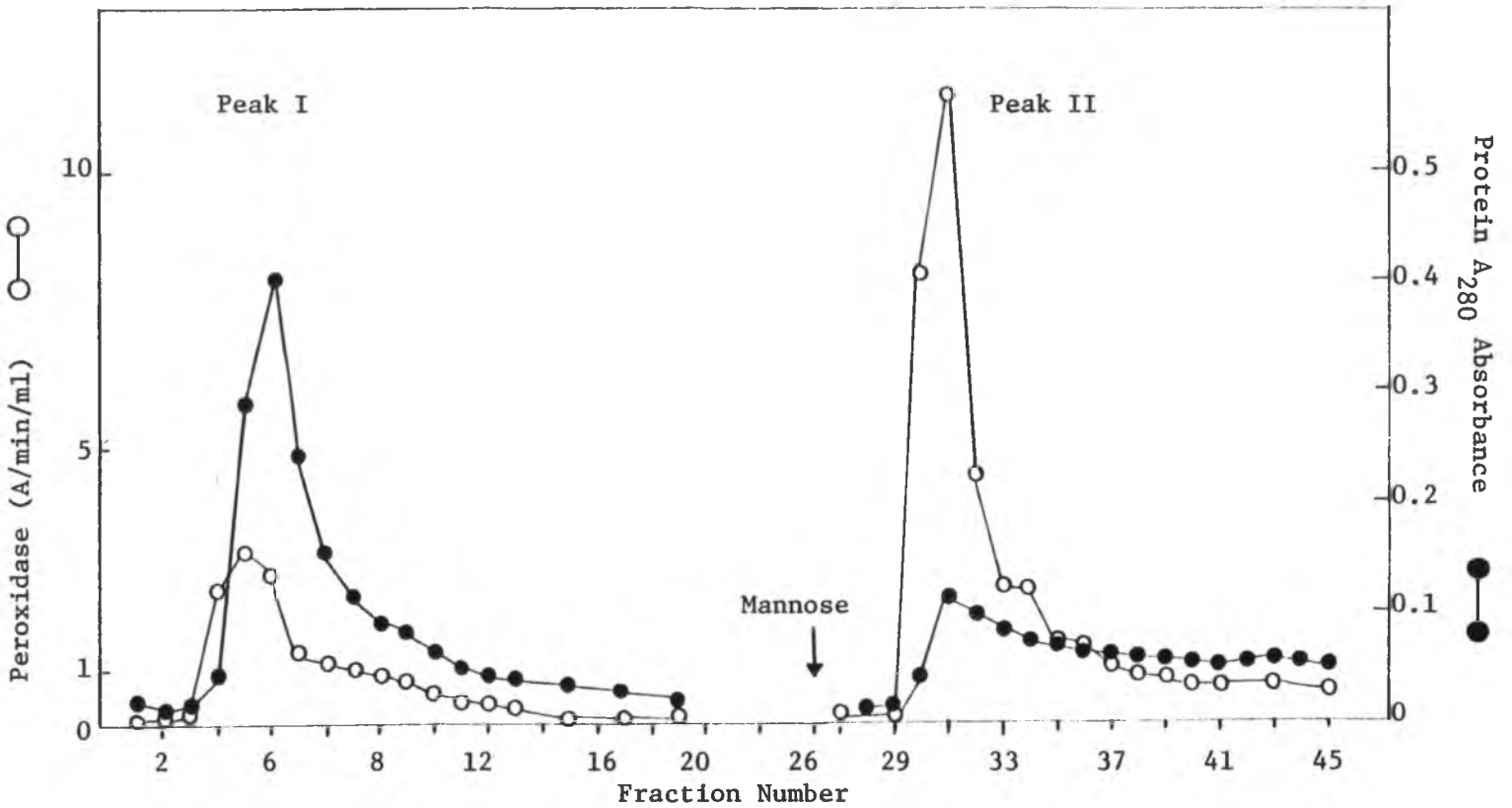


Figure 9. Purification of maize leaf peroxidase by affinity chromatography on Con A Sepharose 4B.

Table 8. Peroxidase activity of Hi27 purified by Con A affinity chromatography.

Fraction	Volume	Peroxidase Activity	Protein	Total Peroxidase Activity	Specific Activity
	--ml--	--A/min/ml--	--A280nm--	--A/min--	--A/min/A280nm--
$(NH_4)_2SO_4$ fraction (loaded)	1.0	66.85	4.65	66.85	14.38
Peak I (wash)	5.33	2.36	0.345	12.55	6.84
Peak II (eluate)	5.33	6.63	0.087	35.27	76.21

76.21. By the Con A chromatography method, a 5.30 fold purification of the maize peroxidases (Peak II) was achieved.

Although the glycoprotein nature of peroxidase has been previously reported (Shannon et al. 1966), recovery of selected maize isozymes by Con A chromatography is a rare example of the glycoprotein nature of peroxidases being used as a means of purification. The recovery value of Peak II (53%) was lower than that of HRP (73%) by Brattain et al. (1976), however the effective purification obtained here was equivalent.

Enzymes from two peaks I and II were concentrated with N_2 flow and were electrophoresed on acrylamide gels (Figure 10). The Peak I fraction consisted of cathodal peroxidase Px1 and a trace of Px3, while the Peak II fraction consisted of anodal peroxidases Px3 and Px7. Px6 was slightly visible in the original solution but could not be detected even after concentration. This result indicates that Px3 and Px7 peroxidases have a specific interaction with Con A. Since Con A has a general affinity for mannose-containing carbohydrates (Reimann and Schonbaum 1978), it is suggested that Px3 and Px7 peroxidases are mannose-containing glycoproteins.

Dye-matrix ligand chromatography was performed in another step of peroxidase purification, using dye-matrix kits described in methods section 3.4. This method has been used successfully for the purification of many enzymes, although no reports of peroxidase purification were found. Some of the dye matrices were effective in separating Px3 and Px7. The result of the dye-matrix chromatography screening is shown in Table 9. $(NH_4)_2SO_4$ fractioned enzyme solution

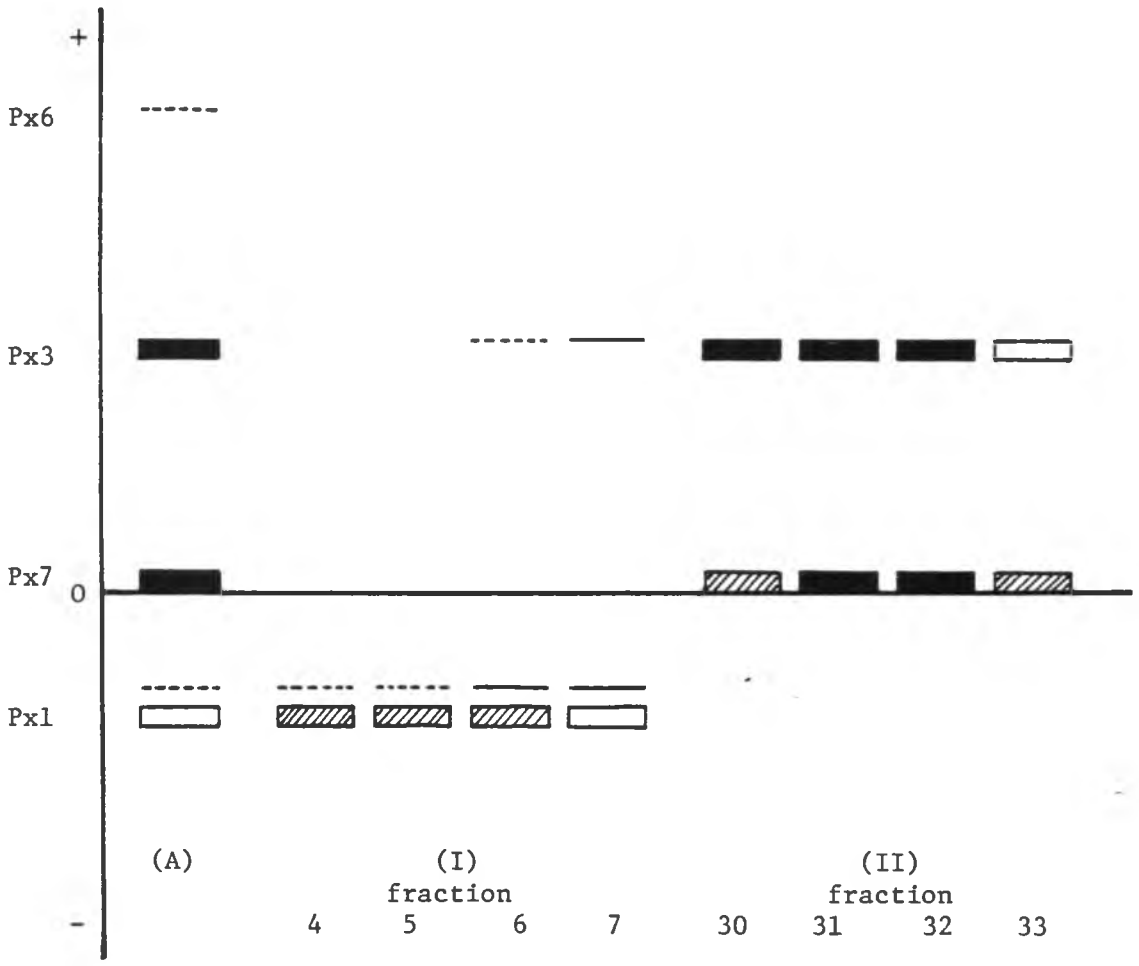


Figure 10. Zymogram of peroxidase isozymes in Con A Sepharose 4B fractions. (A) is the complete enzyme solution loaded on Con A Sepharose 4B. (I) and (II) are peak fractions washed and eluted from Con A Sepharose 4B (cf. Figure 9).

Table 9. Dye matrix ligand chromatography screening of maize peroxidases.

Item	Control	Dye Matrix Ligand Blue A	Orange A	Green A
1. Load enzyme[1)]	19.44	24.30	24.30	24.30
2. Wash enzyme[1)]	13.23	11.18	19.06	13.85
3. Eluate enzyme[1)]	5.29	13.57	9.75	15.28
4. Load protein[2)]	0.89	1.12	1.12	1.12
5. Wash protein[2)]	2.17	1.70	1.74	1.64
6. Eluate protein[2)]	0.45	0.24	0.17	0.23
7. Specific activity of load enzyme[3)]	21.78	21.70	21.70	21.70
8. Specific activity of wash enzyme[3)]	6.08	6.57	10.43	8.26
9. Specific activity of eluate enzyme[3)]	11.75	55.42	57.35	56.63
10. Purification[4)]	X 0.50	X 2.54	X 2.63	X 3.01
11. Yield in %[5)]	95.27	101.4	118.6	115.0

1) A/min.
2) A280nm.
3) A/min/A280nm.
4) Specific activity of eluate/Specific activity of load enzyme (9/7).
5) Expressed as enzyme activity recovered in wash and elute fraction relative to the activity loaded (2+3/1 in %).

was loaded on the four dye-matrix columns, including a Sepharose control. Blue A, Orange A, and Green A yielded increased specific activity in dye matrix-bound fractions. Activities in bound fraction were more than 50% in Blue A and Green A, but low in Orange A and control. In addition, the total activity recovery was 100%, except for the control.

The two fractions, washing or elution, were concentrated and electrophoresced using the original enzyme solution as a control (Figure 11). Blue A and Green A fractions showed a clear separation of peroxidase isozymes. Px3 was washed through the columns by the buffer, while Px7 was bound to the dye matrix and was obtained only by elution with 1.5 M KCl. Orange A and control did not affect a separation of the two isozymes. Based on these results, Blue A was chosen as the preferred dye matrix chromatography for Px7 and Px3 purification.

Blue Sepharose Cl-6B (Sigma Co.) has the same properties as Blue A dye in the kit, and was prepared as described in methods section 3.4. The concentrated fraction of Peak II of Con A was applied to a column of Blue Sepharose and two peaks were obtained (Figure 12). The first Peak (Peak A, fractions 3-6) had higher activity and a high 280 mm absorbance. The second peak (Peak B, fractions 6-8) had medium level activity and very low 280 mm absorbance. Proportions of activities in the two peaks are shown in Table 10. Total enzyme recovery of activity in the two peaks was 79.7%. Peak B had a 2.85-fold higher relative activity than Peak A. Concentrated enzymes from each peak were electrophoresced and Px3

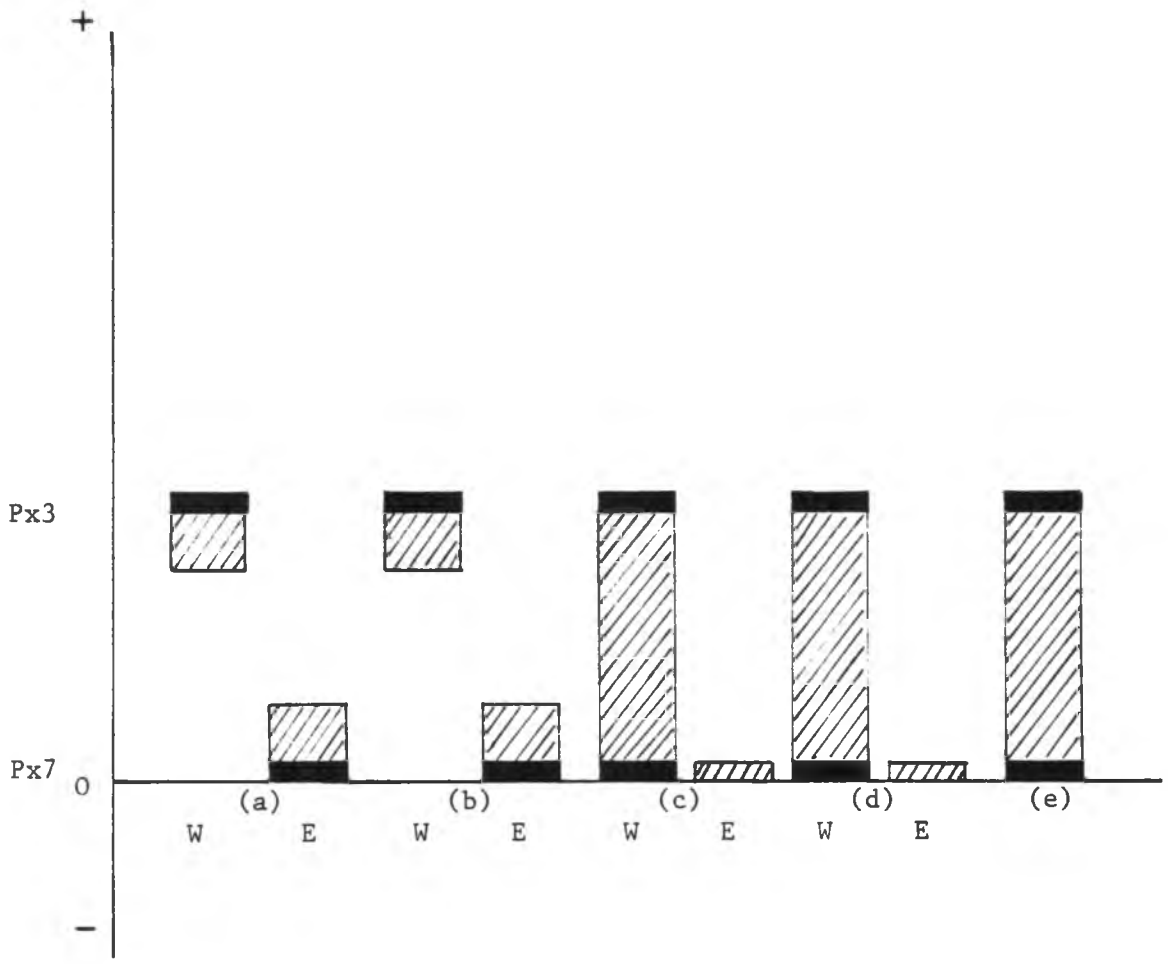


Figure 11. Zymogram of peroxidase isozymes in dye-matrix legand chromatography screen. (a) Blue A, (b) Green A, (c) Orange A, (d) control, and (e) enzyme loaded. W is for washed fraction and E is for eluted fraction.

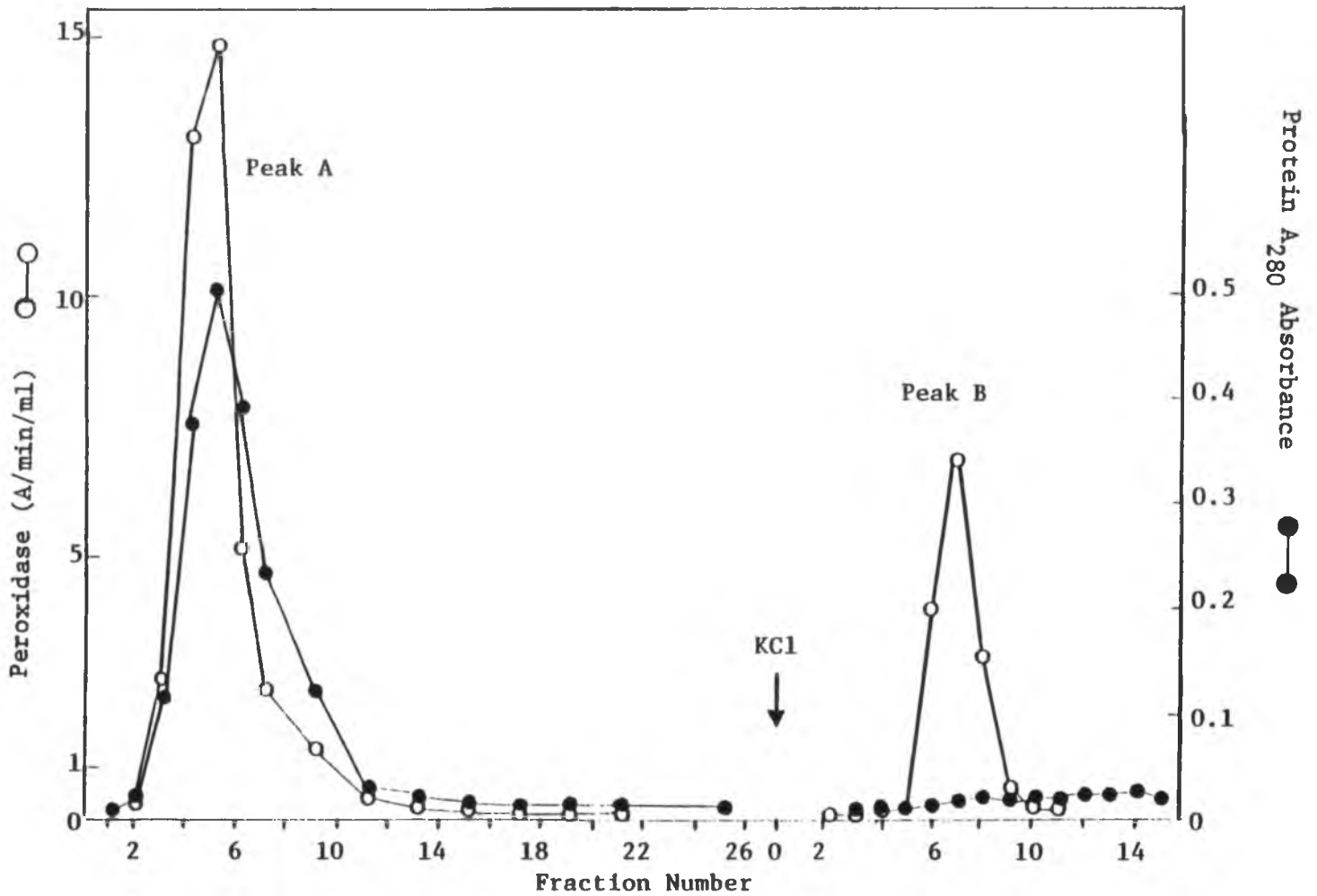


Figure 12. Separation of maize leaf peroxidase by affinity chromatography on Blue Sepharose CL-6B.

Table 10. Peroxidase activity of Hi27 purified by Blue Sepharose CL-6B (cf. Fig. 12).

Fraction	Volume	Peroxidase Activity	Protein	Total Peroxidase Activity	Specific Activity
	--ml--	--A/min/ml--	--mg/ml--	--A/min--	--A/min/mg--
Enzyme loaded	1.0	104.80	1.070	104.80	97.94
Peak A (wash)	1.4	21.70	0.350	30.38	62.00
Peak B (eluate)	0.7	8.95	0.032	6.27	279.20

and Px7 were seen on the zymogram (Figure 13). Px7 was from the Blue Sepharose bound fraction (Peak B), while Px3 was from the buffer washed fraction (Peak A).

The mechanism of interaction between Blue A dye and proteins is not clear, but it has been reported that the structural components of Blue A dye have strong interaction with a substrate or cofactor binding site. Blue A chromatography has been used for purification of various enzymes including 25 oxidoreductases and 15 kinases from many species. In maize, R enzyme of sweet corn was purified using blue dextran coupling, a method similar to Blue A ligand (Marshall 1970).

It is thus shown that two types of affinity chromatography, Con A Sepharose 4B and Blue Sepharose 6B, were effective in separating and purifying Px3 and Px7 isozymes. The recovery of each enzyme activity after purification, however, was not very high.

4.2.2 Km Determinations of Allozymes at Px3 and Px7.

To compare the activity of Px3 and Px7 isozymes on different substrates, the Michaelis Constant (Km) was determined for 5 maize lines, Hi27, B37, F**, SF, and T_{24}. Km values are convenient in determining substrate specificities of enzymes whose purities are not the same. Px3 and Px7 enzymes were separated by Blue Sepharose 6B for use in these studies. Lineweaver-Burke plots, relating 1/velocity to 1/substrate concentration, were constructed to determine Km values. The velocity term used in these plots is the

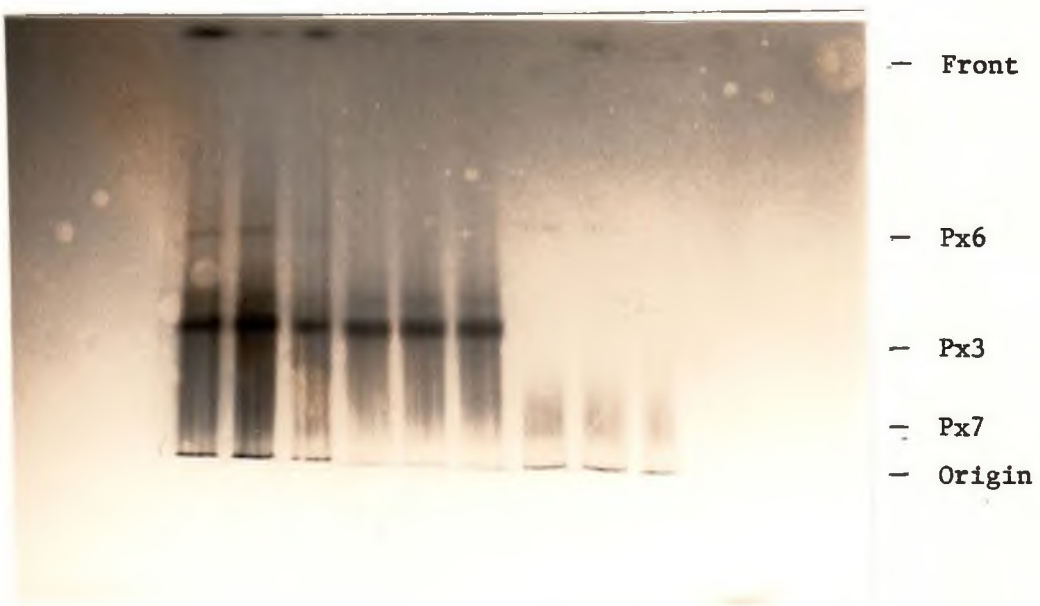


Figure 13. Zymogram of Con A fraction II through column of Blue Sepharose CL-6B: (a) Con A fraction II (enzyme loaded), (b) washed by Tris-HCl buffer and (c) eluted by KCl in buffer.

peroxidase activity, A/min/ml enzyme. The substrates examined included H_2O_2, o-dianisinide, ferulic acid and caffeic acid.

O-dianisidine is a di-phenol artificial dye which is commonly used as a peroxidase substrate. Caffeic acid and ferulic acid are naturally occurring substrates, known as lignin precursors, and have similar structures except for a methoxy group difference.

Inbred lines that contained allozymes of each locus, Px3 and Px7, were used as enzyme sources. The lines and their allozymes are as follows:

Hi27:	Px3-F,	Px7-S
B37:	Px3-S,	Px7-S
$\overline{SF}$:	Px3-$\overline{SF}$,	Px7-S
F**:	Px3-F**,	Px7-S
T_{24}:	Px3-F, S, S/F,	Px7-F

Lineweaver-Burke plots of these allozymes are presented in Appendix 10-14.

Km values for allozymes of the inbred lines examined using o-dianisidine are presented in Table 11a. There were no significant differences in Km values among allozymes within each locus or between Px3 and Px7. Km values for caffeic acid are shown in Table 11b. Px3-F and Px3-F** had lower Km values than other allozymes examined, however, differences were not significant. As with o-dianisidine, Km values of two loci had no significant difference. Km values for Px3 and Px7 with ferulic acid were found to be

Table 11. Km determinations of peroxidase isozymes.

Line	Px3	Km	Px7	Km
		-----M-----		-----M-----
		a) o-dianisidine		
Hi27	F/F	3.95×10^{-5}	S/S	4.46×10^{-5}
B37	S/S	6.46×10^{-5}	S/S	2.57×10^{-5}
F**	F**/F**	5.83×10^{-5}	S/S	---
SF	SF/SF	4.85×10^{-5}	S/S	3.31×10^{-5}
T24	S/S,F/F,S/F	7.90×10^{-5}	F/F	2.88×10^{-5}
	Mean	5.49×10^{-5}	Mean	3.30×10^{-5}
	Mean difference	8.58×10^{-4}M, t = 2.357* (P = .05)		
		b) Caffeic acid		
Hi27	F/F	0.63×10^{-4}	S/S	0.87×10^{-4}
B37	S/S	3.60×10^{-4}	S/S	3.08×10^{-4}
F**	F**/F**	0.84×10^{-4}	S/S	7.88×10^{-4}
SF	SF/SF	3.70×10^{-4}	S/S	2.28×10^{-4}
T24	S/S,F/F,S/F	1.13×10^{-4}	F/F	1.66×10^{-4}
	Mean	1.98×10^{-4}	Mean	3.15×10^{-4}
	Mean difference	1.17×10^{-4}M, t = 0.826		
		c) Ferulic acid		
Hi27	F/F	11.42×10^{-4}	S/S	3.77×10^{-4}
B37	S/S	6.79×10^{-4}	S/S	5.98×10^{-4}
F**	F**/F**	10.23×10^{-4}	S/S	4.44×10^{-4}
SF	SF/SF	27.44×10^{-4}	S/S	3.92×10^{-4}
T24	S/S,F/F,S/F	12.21×10^{-4}	F/F	7.07×10^{-4}
	Mean	13.63×10^{-4}	Mean	5.04×10^{-4}
	Mean difference	8.58×10^{-4}M, t = 2.357* (P = .05)		

significantly different ($p = .05$) between two loci (Table 11c), but not among alleles. In Px7, average Km was 5.04×10^{-4} M, while in Px3 it was 13.62×10^{-4} M.

The results indicate that Px7 had a higher affinity for ferulic acid than Px3, while both Px7 and Px3 had similar affinities to caffeic acid and o-dianisidine. Both of the naturally occurring substrates caffeic acid and ferulic acid, are known as lignin precursors (Stafford 1963). In a model system with peroxidase and ferulic acid, lignin-like products were found (Stafford 1963). Ferulic acid and p-hydroxylcinnamic acid were reported as the main components of maize hydrolyzed lignin (Kuc and Nelson 1964). Ferulic acid appears to be a lignin precursor in maize, but the importance of caffeic acid as a maize lignin precursor has not been clarified.

The difference in Km values for ferulic acid may indicate that the two isozymes, Px3 and Px7, have different physiological functions in the cell. Px7 appears to utilize ferulic acid more effectively than Px3 or other peroxidase isozymes and therefore makes a greater contribution to lignin formation in maize tissues. Px7 was observed to have a high relative activity to eugenol, another lignin precursor (Brewbaker and Hasegawa 1975). A large proportion of Px7 activity is reported to be located at the cell wall where active lignin formation occurs. The Px7 examined here was derived from cytoplasmic fractions (Brewbaker and Hasegawa 1975), however, cell wall Px7 is expected to have the same catalytic properties as cytoplasmic Px7. To obtain additional evidence of Px7's catalytic advantage in lignin biosynthesis, it is suggested for future study that the affinity of

cytoplasmic and wall-bound Px7 and Px3 with various naturally occurring lignin precursors such as eugenol and p-coumaric acid be examined using methods described in this section.

Allozymes of the two enzymes, Px3 and Px7, did not have any signfiicant difference in Km values. Allozymes Px3-F, Px3-S, Px7-F and Px7-S are widely distributed in many inbred lines, while Px3-F** and Px3-$\overline{SF}$ are originally found in the Puya race, not found in common inbred lines. Since there were no differences of Km values between the common allozymes and the unusual ones, it appears that the catalytic activity of Px3-F** and Px3-$\overline{SF}$ allozymes do not vary from the ability of common allozymes. Other allozyme characteristics, however, such as optimum pH and thermostability, may differ. Felder et al. (1973), for example, found that two allozymes of alcohol dehydrogenase had distinct heat labilities but had similar Km, pH optima and molecular weights. The differences of mobility on electrophoresis among allozymes of Px3 and Px7 observed here may be due to simple amino acid substitutions which do not affect catalytic abilities.

4.2.3 Molecular Weight of Px3 and Px7.

The molecular weight determination study was conducted jointly with Dr. E. H. Liu during his stay at the University of Hawaii in Fall 1979 and Spring 1980 (Liu, Nagai and Brewbaker 1981). Molecular weight of peroxidase isozymes was determined using Sephadex G75 in 0.05 M phosphate buffer pH 7.0, which had been calibrated with known molecular weight standards; Ribonuclease A (MW 13,700),

Chymotrysinogen A (MW 25,000), and Ovalbumin (MW 45,000) (Figure 14). By electrophoresis of aliquots from Sephadex fractions, the peak fractions of each isozyme were determined and elution volumes were obtained. At the same time corresponding isozymes were determined. Molecular weights of the isozymes found in the present study ranged between 33,000 and 72,000. MW of Px3 was 38,000, which was similar to the MW of isozymes Px1, Px5, Px6 and Px9, while Px7 had a MW of 72,000.

Px7 was eluted in the fractions very close to the void volume in Sephadex G-75 (the void volume of G-75 contains molecular weights greater than 75,000) and Px7 often streaked on acrylamide gels. These facts raise the possibility that Px7 may be bound to large cellular structures such as cell walls or membranes as noted by Hasegawa (1974) indicating that the estimated MW of 75,000 may be inaccurate. To examine whether the MW of Px7 is correct, larger sieve chromatography Sephadex G-100 and Sephadex G-200 were utilized. A crude enzyme extract of inbred T55 (Px7-F, Px3-5) was used with Blue dextran (MW 2,000,000) as a standard. A clear difference in peaks of Px3 and Px7 was obtained from Sephadex G-100 and G-200 chromatography (Figure 15). Px7 was retained within the pore structure of the molecular sieve gels of Sephadex G-100 and G-200 (Table 12). Even though Px7 has a largest maize isozyme molecular weight (MW 72,000), this result showed that it did not bind to other large moleculars in the cell. Thus, it was concluded that the

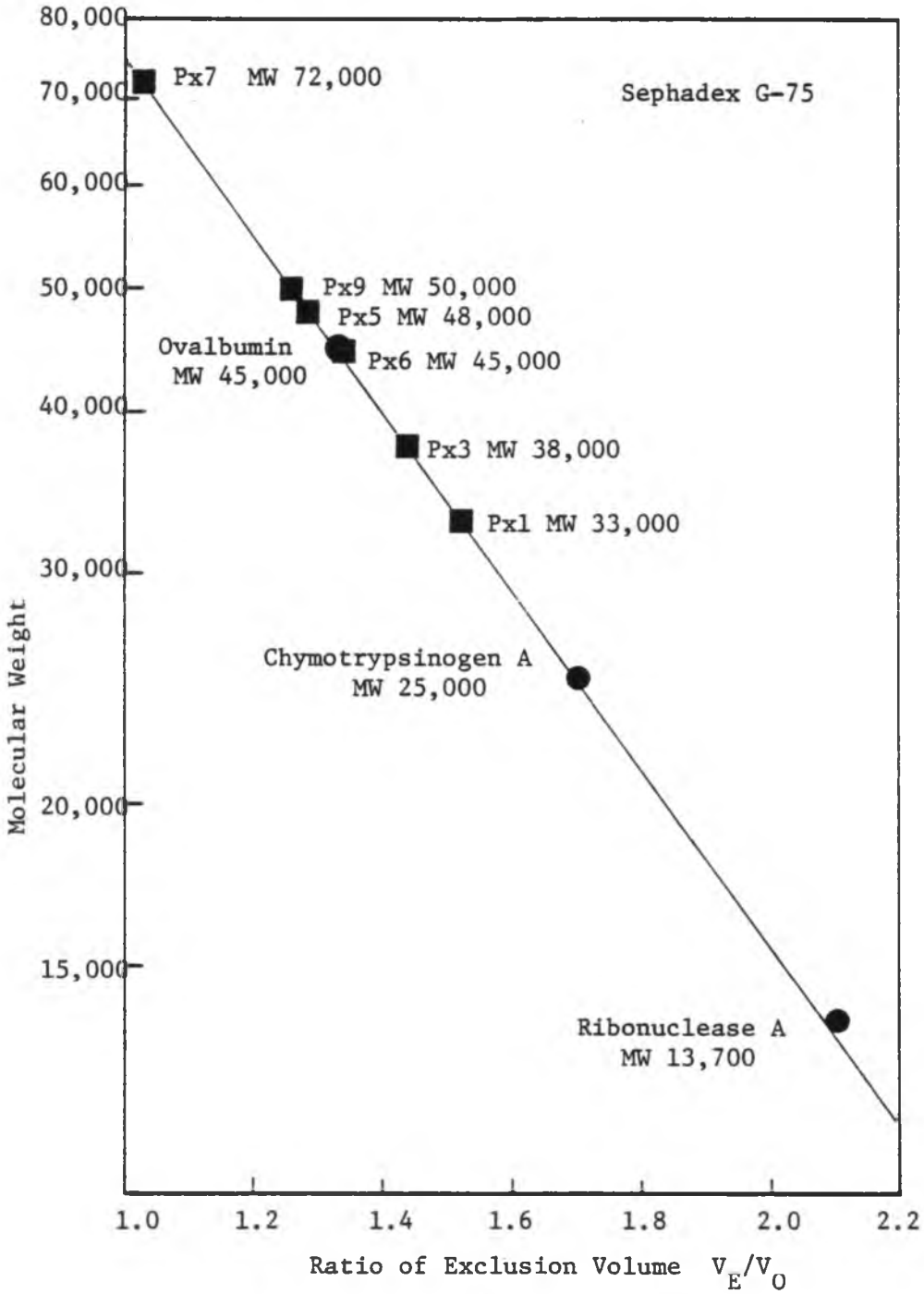


Figure 14. Estimation of molecular weight for peroxidase isozymes of maize using Sephadex G-75 column chromatography.

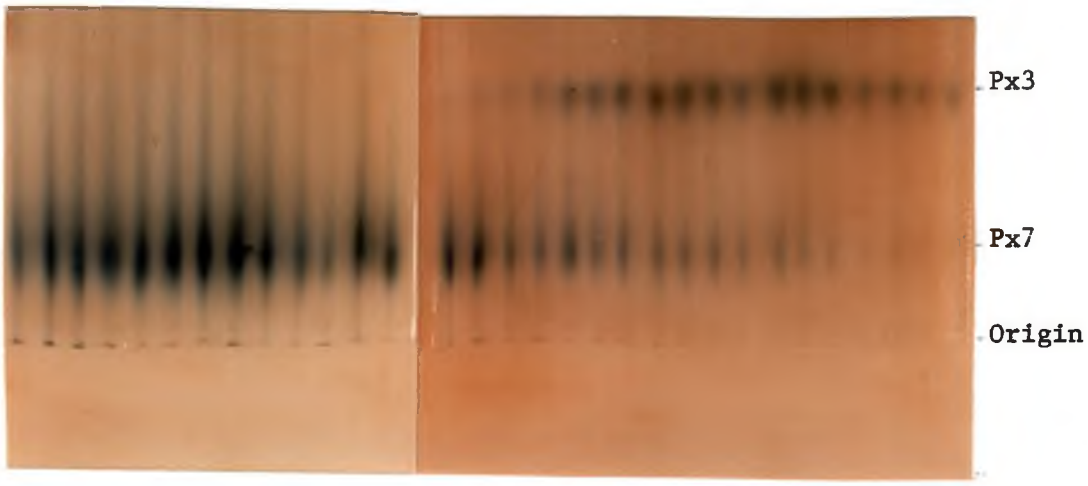


Figure 15. Zymogram of peroxidase isozymes from Sephadex G-200 column chromatography. Each fraction contains 0.1 ml eluate.

Table 12. Elution of Px7 from Sephadex molecular sieve chromatography column.

Sephadex Pore Type	Exclusion MW	Rate of elution volume / void volume (Ve/Vo)
G-75	75,000	1.05[1)]
G-100	100,000	1.22[1)]
G-200	200,000	1.50

1) Data by Dr. Liu.

estimated MW was correct and not a result of binding to other molecules. MW of Px7 was almost twice as large as that of Px3.

The molecular weights of peroxidase isozymes from various species including pea, horseradish and turnip have been determined by others (Gibson and Liu 1978, Shannon et al. 1966). The inheritance of peroxidase isozymes in these plants, however, is unknown. Inheritance pattern studies in maize, described in Section 4.1, indicate that Px3 and Px7 are monomers. Because Px7 is a monomer, and not a dimer, other factors such as differences in tertiary structure or sugar moiety may account for the observed MW difference.

The biochemical heterogeneity of two major isozymes of maize, Px7 and Px3, was determined from molecular weights and Km values. It is still unclear, however, whether the large molecular weight of Px7 is related to its high affinity for ferulic acid. Future research to determine the relationship between molecular weight differences and specific substrate affinities may include definition of the physical structures of maize peroxidases with purified isozymes. Carbohydrate composition, amino acid composition and absorption spectra for heme content will also help clarify the structure of these isozymes.

4.3 Peroxidases in Morphological Mutants

The roles of peroxidases during plant development have been studied by various researchers as described in the Literature Review. It is well known that peroxidases are involved in degradation of IAA which directly regulates plant growth. Also, peroxidase involvement in lignin formation and oxidation of phenolic compounds may be important in various developmental stages of plants. On the other hand, morphological mutants that affect phenotypes of leaves and plant stature during development are expected to have metabolisms different from normal plants including growth hormone level and enzyme activity. Some dwarf mutants have been reported to have a lower level of auxin (Overbeek 1938) or gibberellic acid (Phinney 1956). Greater than normal peroxidase activity has been reported in dwarfs of many species (Evans 1968, Birecka and Galston 1970, Schertz et al. 1971).

In maize, the genetic control of peroxidases is clear and substrate specificity difference among isozymes suggested the difference of physiological role in plants (Brewbaker and Hasegawa 1975). Thus, to investigate the physiological roles of peroxidase isozymes in the developmental stages of maize, a study of leaf peroxidases in morphological mutants was conducted.

4.3.1 Morphology of Mutants.

Fourteen morphological mutants of maize that have distinct phenotypes of leaves, stems and stature during development were chosen for study (Table 13). These mutants were selected following preliminary screening by Brewbaker and Hasegawa (unpublished). The

Table 13. Maize morphological mutants examined for peroxidase activity.

Name	Mutant Symbol	Gene Location		Phenotype
Dwarfs				
dwarf	d	3-18	*	plant andromonoecious, short, compact
dwarf-tiny	d-tn	3-18	*	allele of d, tiny
brachytic	br	1-81	**	short internodes, short plant, leaves normal
brachytic 2	br2	1L	**	like br, but less dwarfed
crinkly leaf	cr	3-0		plant short, crinkled leaves
nana	na	3-86	**	very short, with dwarfed leaves short
nana 2	na2	5-	**	like nana
pygmy	py	6-68	**	plant dwarfed, short leaves, pointed
Aborted leaf				
Ragged	Rg	3-48		chlorotic tissues between veins, hole and torn appearance
slashed leaf	sl	7-50		leaves slit longitudinally by necrotic streaks
Tumorous tissue				
Knotted	Kn	1-127	*	tumorous growth along vascular bundles of leaves
Grassy mutant				
Corn grass	Cg	3-31	*	narrow leaves, extreme tillering
grassy tiller	gt	-		numerous basal branches
Others				
lazy	la	4-60	**	prostrate growth habit

* Gibberrelic Acid responding
** Non Gibberrelic Acid responding

mutants included dwarf, aborted leaf, tumorous tissue and grassy mutants, all as isogenic conversions of Hawaii inbred Hi27. The leaf length and plant height were measured in mature plants (Table 14). Seven recessive dwarf mutants, d, d-tn (allelic mutant) to d, br, br2, na and na2 varied in their phenotype and degree of dwarfism. Mutant plants na, na2 and d are shown in Figure 16. Two mutants, d and d-tn are gibberellic acid (GA) responding, while others are known as non-GA responding. The dwarfness of d leaves was much smaller than that of plant height, while py showed less dwarfing of stems than leaves. Mutant br and br2 are classified as semi-dwarf since their plant height and leaf length were close to those of normal (Table 14). Both na and na2 showed severe dwarfness, with plant heights less than half of Hi27. Not only plant height and leaf length but shape and leaves in these dwarf plants were different. Mutants br, br2, d and d-tn showed normal healthy leaves, while na, na2 and py had rough surfaced leaves with pointed leaf tips. Because of the variable phenotypes of dwarf plants, different mechanisms of dwarfism may be expected.

Aborted leaf (Rg, sl), grassy (gt, Cg) and tumorous (Kn) mutants also showed some dwarfism (Table 14). Mutant gt did not have significant difference from control in plant height and leaf length, however, Cg showed dwarf phenotype with its main phenotype being extensive tillering and thin leaves. Mutant sl had small leaves though plant height was close to control inbred Hi27 (Figure 17). Rg showed extreme dwarfism in leaves that were only 30% the length of Hi27 (Figure 17). Also Rg and sl had small and narrow leaves with

Table 14. Leaf length and plant height of morphological mutant maize at tasseling stage.

Mutant	Leaf Length		Plant Height	
	--cm---		---cm---	
Hi27	93.4[1]	a[2]	199.4[3]	a[2]
gt	85.6	ab	197.4	a
br	-	-	141.0	bc
br2	84.0	ab	159.6	b
Kn	66.2	c	135.2	c
Cg	65.2	c	155.4	bc
d	65.0	c	110.4	d
sl	61.8	c	166.4	b
na	58.6	cd	77.2	e
na2	50.4	d	89.8	e
py	50.0	d	139.4	c
Rg	31.8	e	145.3	bc
d-tn	-	-	51.5	f
	(-30.0)			

1) Means of 5 samples of the 7th leaf
2) Means followed by the same letter are not significantly different at the 5% level according to Duncan's multiple test.
3) Means of 10 samples.

na(right) and na2

d

Figure 16. Normal and dwarf plants of maize at the 4 week stage.

Rg

sl

Figure 17. Rg and sl plants of maize at the 5 week stage.

necrotic parts on leaves. Overall, leaf length in mutants ranged from 30 to 92% of normal, while plant heights were 26-99% normal, with dwarfing found in both leaves and stems.

4.3.2 Tissue Source of Peroxidases in Mutant Maize.

Maize stems and leaves were the most feasible sources of peroxidases. Preliminary experiments to detect peroxidase in several maize mutants (Brewbaker and Park 1977, unpublished) showed that the difference in peroxidases between normal and mutant maize was larger and error variance lower in leaves than in internodes. Therefore, leaf tissues were chosen as the peroxidase source for this study. Until the age of 4 weeks, the plant height of maize reflects only leaf length, as growing apex is very near the ground.

The age of leaves to be harvested must be specified to conduct experiments of peroxidases at the different developmental mutants. Peroxidase activity in leaves of different ages is known to be different (Hamill 1970). Therefore, a preliminary experiment was conducted to determine the source of enzyme for analysis. Leaves of Hi27 inbred were numbered from the oldest to the youngest at the 6 week stage and measured for peroxidase activity (Figure 18). Older leaves showed higher peroxidase activity than younger leaves, both in total activity and specific activity. The leaves at positions lower than 8 could not be harvested due to senescence. Peroxidase from leaves #13-15 appeared to be the least variable, therefore, the 3rd open leaf from the top (in this case #14) was chosen as the standard source of peroxidase in this study. The higher peroxidase activity in

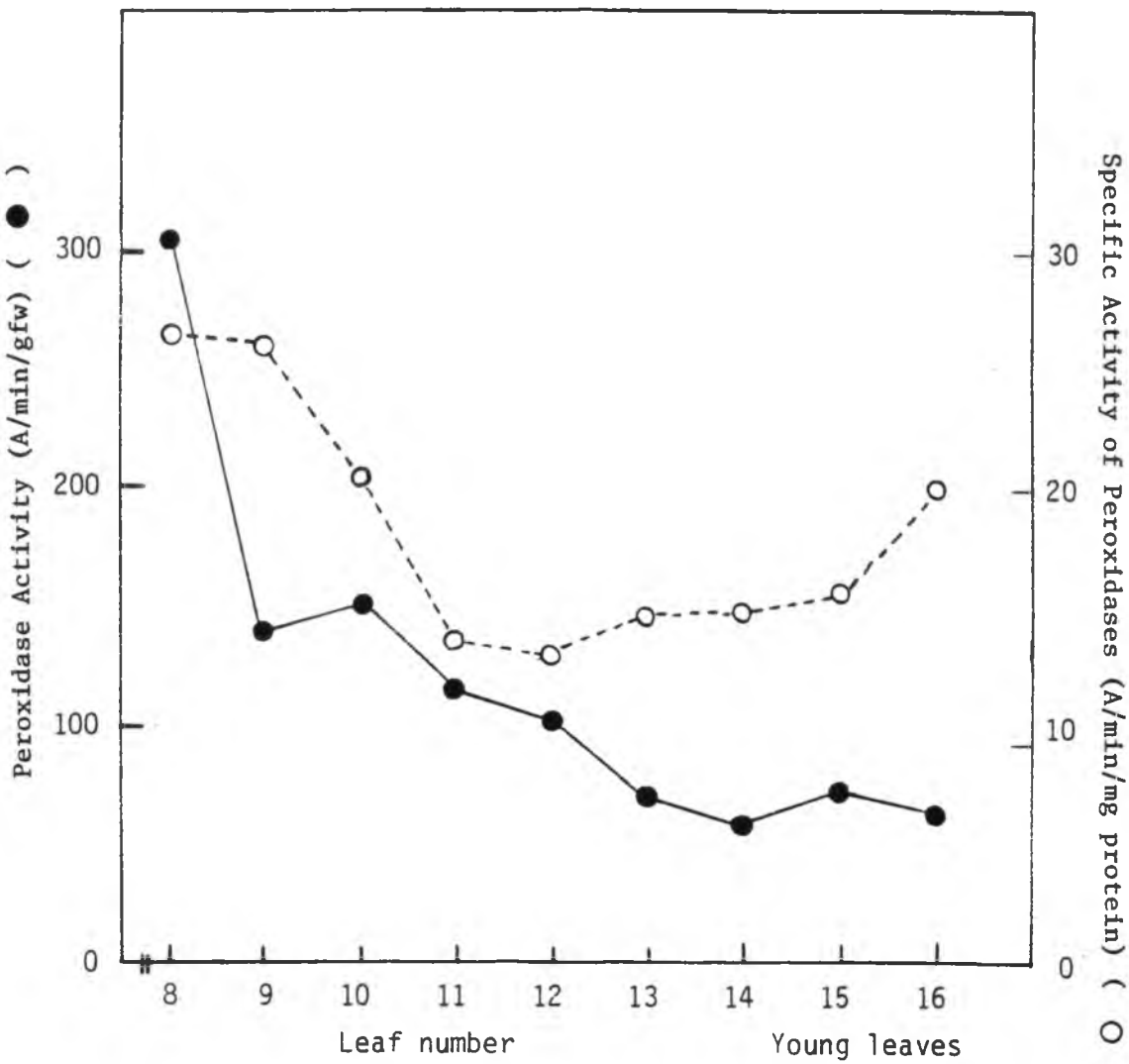


Figure 18. Peroxidase activity and leaf location of 6 week Hi27 plant.

older leaves is possibly due to senescent tissues which showed higher lignification and necrosis. Also, rust (Puccinia sorghi) infection, observed in lower positioned leaves, might be one of the reasons for high activity (Kim et al. 1978).

4.3.3 Peroxidase Activity in Dwarf Mutants.

Plant height and leaf peroxidase activity were examined in the fourteen maize mutants following methods described in section 3.6. Hi27 was used as control throughout the experiments. Growth curves of these mutants are shown in Figure 19ab. Until the 4 week stage, plant heights corresponded to leaf length since meristem is underground. Mutant br and br2 showed similar growth curves which compared with Hi27 until 4 weeks, but between 5 and 8 weeks plant heights were reduced. The na mutant started to display stunted phenotypes as early as the two week stage. After 5 weeks na plant height became constant. Mutant d had a growth curve similar to Hi27, br and br2 except that it started dwarfing at 4 weeks stage. Mutant d-tn was already shorter than Hi27 at the 2 weeks stage and at 5 weeks stage it reached its maximum height. Mutant py started to show dwarfness as early as two week stage (Figure 19b).

In br mutants peroxidases increased relative to control after 4 to 5 weeks of development (Figure 20a). Peroxidase activity in br was 1.6 times higher than that of Hi27 in 4 weeks stage. At the stage (4 weeks) when peroxidase activity increased, the growth rate of br decreased. After 6 weeks peroxidase activity returned to the level of

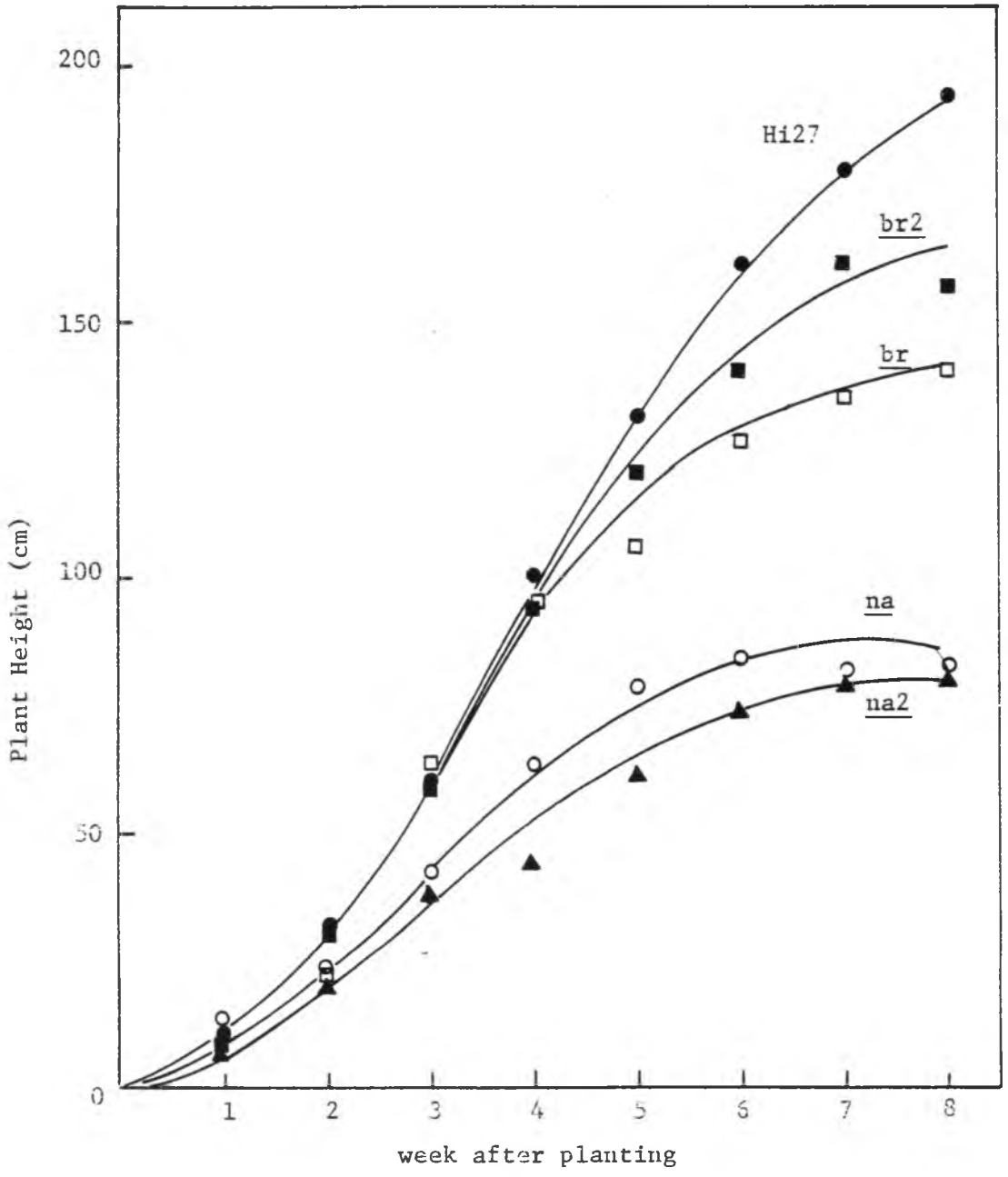


Figure 19a. Plant height of dwarf maize: br(□), br2 (■), na (○), na2 (△), and Hi27 check (●).

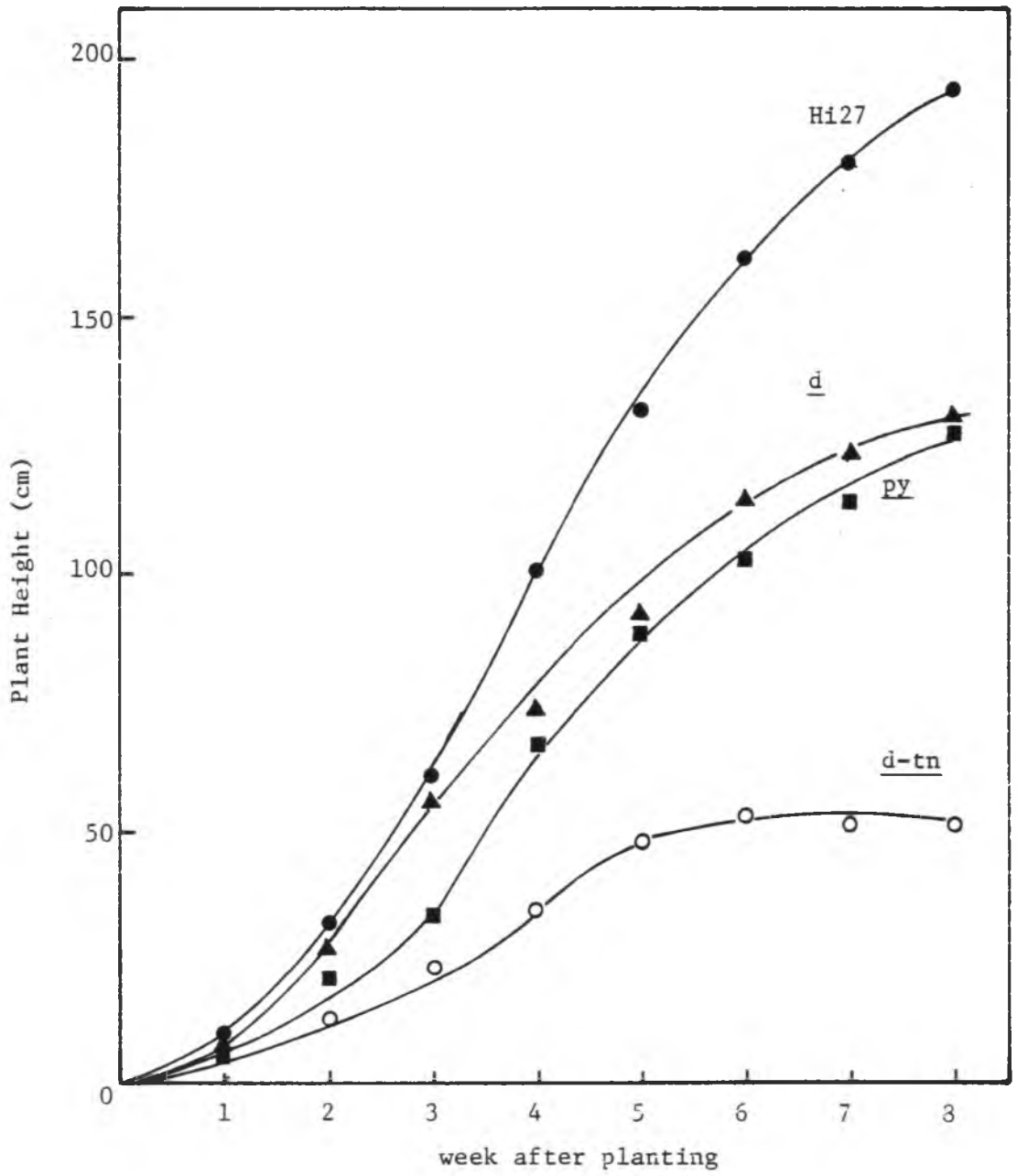


Figure 19b. Plant height of dwarf maize: d(▲), d-tn(○), py(■), and Hi27 check (●).

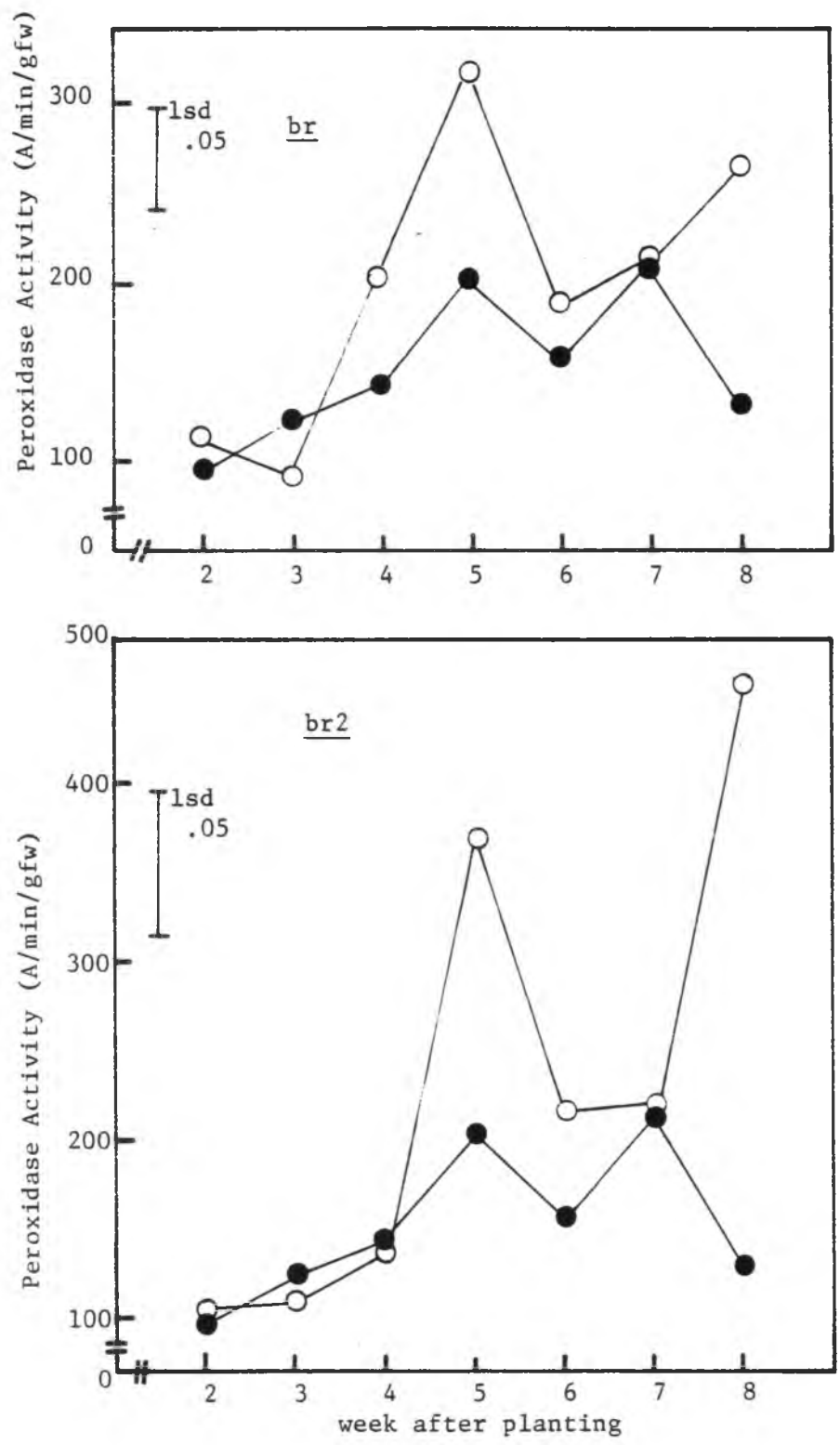


Figure 20. Peroxidase activity during development of dwarf mutants: (a) br, (b) br2 (○), and Hi27 check (●).

Hi27. This mutant was not GA responding (Phinney 1956). A possible function of increased peroxidase in br may be growth retarding IAA oxidation.

In br2 mutants peroxidase activity also increased at the 5 week stage (Figure 20b), when br2 plant growth rate decreased (Figure 19a). However, peroxidase activity decreased between the 6th and 7th weeks and increased again at the 8th week. Mutant br2 has a similar phenotype to br but less leaf dwarfing. Although there was a relationship between the timing of peroxidase increase and plant height decrease, the relationship between dwarfness and peroxidase activity was unclear.

The peroxidase activity in d and its allelic mutant d-tn was not significantly different from that of Hi27 (Figure 21). Only at the 8th week stage in d-tn was an increase of peroxidase activity observed. It has been reported that the application of GA can increase the plant height of d to a normal level (Phinney 1956). McCune and Galston (1959) found no peroxidase decrease in d by application by GA and noted that peroxidase was only one of the biochemical changes by altered growth. Peroxidase increase in d was not significant compared to their reduced plant height, while br and br2 showed higher increase in peroxidase though moderate plant height reduction. If d and d-tn had different leaf density compared to that of control, peroxidase activity expressed per fresh weight may not express their activity correctly. However, comparison of peroxidase activity in specific activity units (/mg protein), still showed no differences between the two lines. Results presented here that the GA-responding dwarfs d

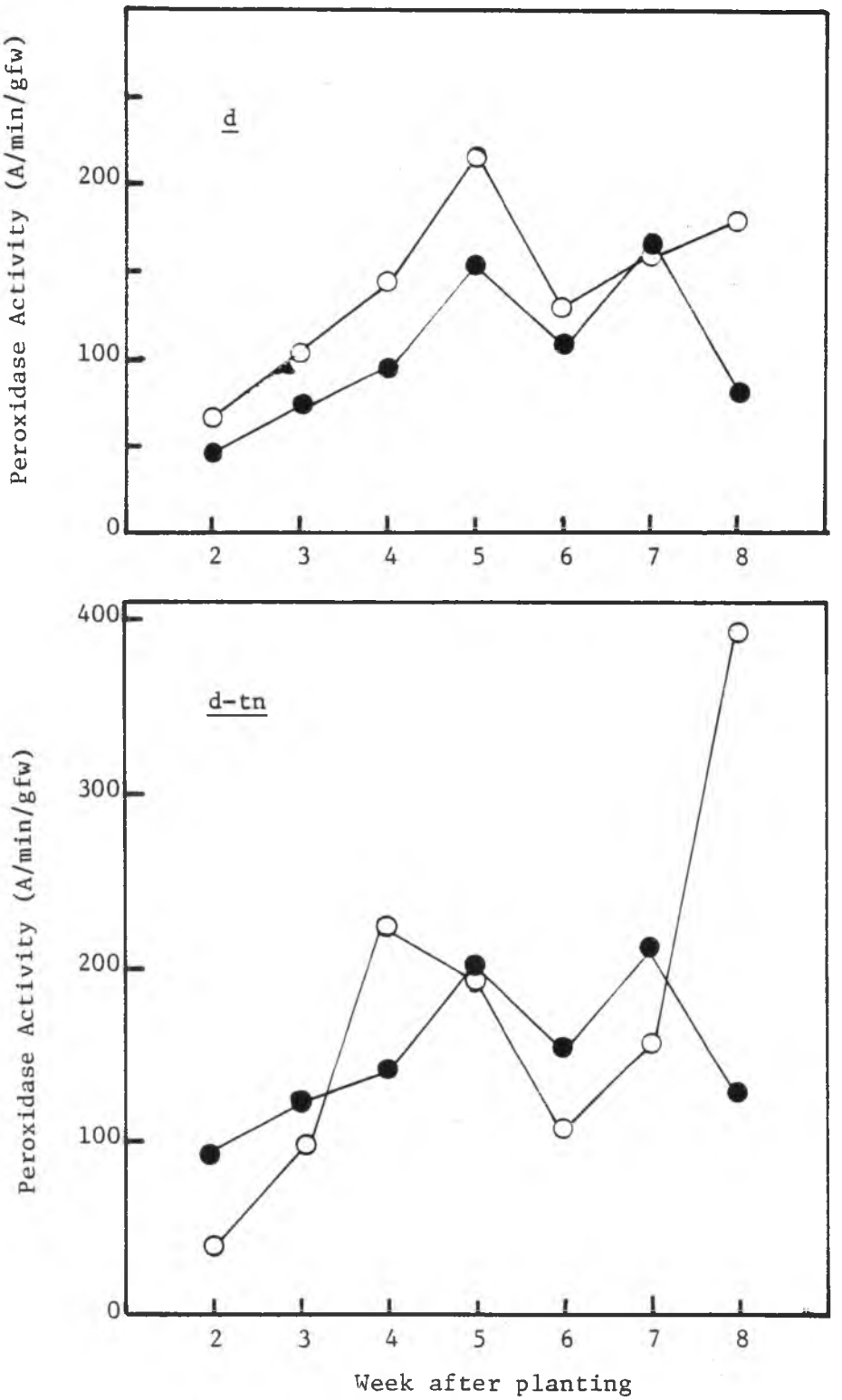


Figure 21. Peroxdiase activity during development of dwarf mutants: (a) d, (b) d-tn (o) and Hi27 check (●).

and d-tn do not have higher peroxidase activities compared to Hi27 also indicated that peroxidase is not the enzyme directly controlling height in d.

Mutant na and na2 showed higher peroxidase activity than Hi27 (Figure 22). Since na and na2 had very similar growth rates and phenotypes (Figure 19a), these two were expected to have similar activities in peroxidase. However, the change of peroxidase levels in these two mutants was different. In na, peroxidases were significantly higher at 7 and 8 weeks of growth. Several papers indicated that na has a lower auxin level than normal (Overbeek 1938) which may cause shortness of plants. In young tissues like mesocotyl and coleoptile of na, difference in peroxidase activity was not found (Shoemaker and Harris 1975), but from results of this study it appears that a high level of peroxidases exists in na leaves. At the 2 week development stage the na phenotype had already become identifiable yet the peroxidase activity was still as low as that of Hi27. The na peroxidase activity began to increase over that of Hi27 after the 7th week. This indicates that high peroxidase activity is turned on after the short phenotype appears. If the reason for shortness in nana is due to a low level of auxin (Overbeek 1938), at least the stage when the dwarf phenotype appears, some other auxin control mechanism must cause it. It may be possible that peroxidase is involved in IAA degradation at a later stage. However, no evidence could be found that leaf peroxidases cause the shortness phenotype of na. Mutant na2 showed an increase of peroxidase activity much earlier than na. At 4 and 5 weeks and again at the 7 and 8 week stage, activity reached more

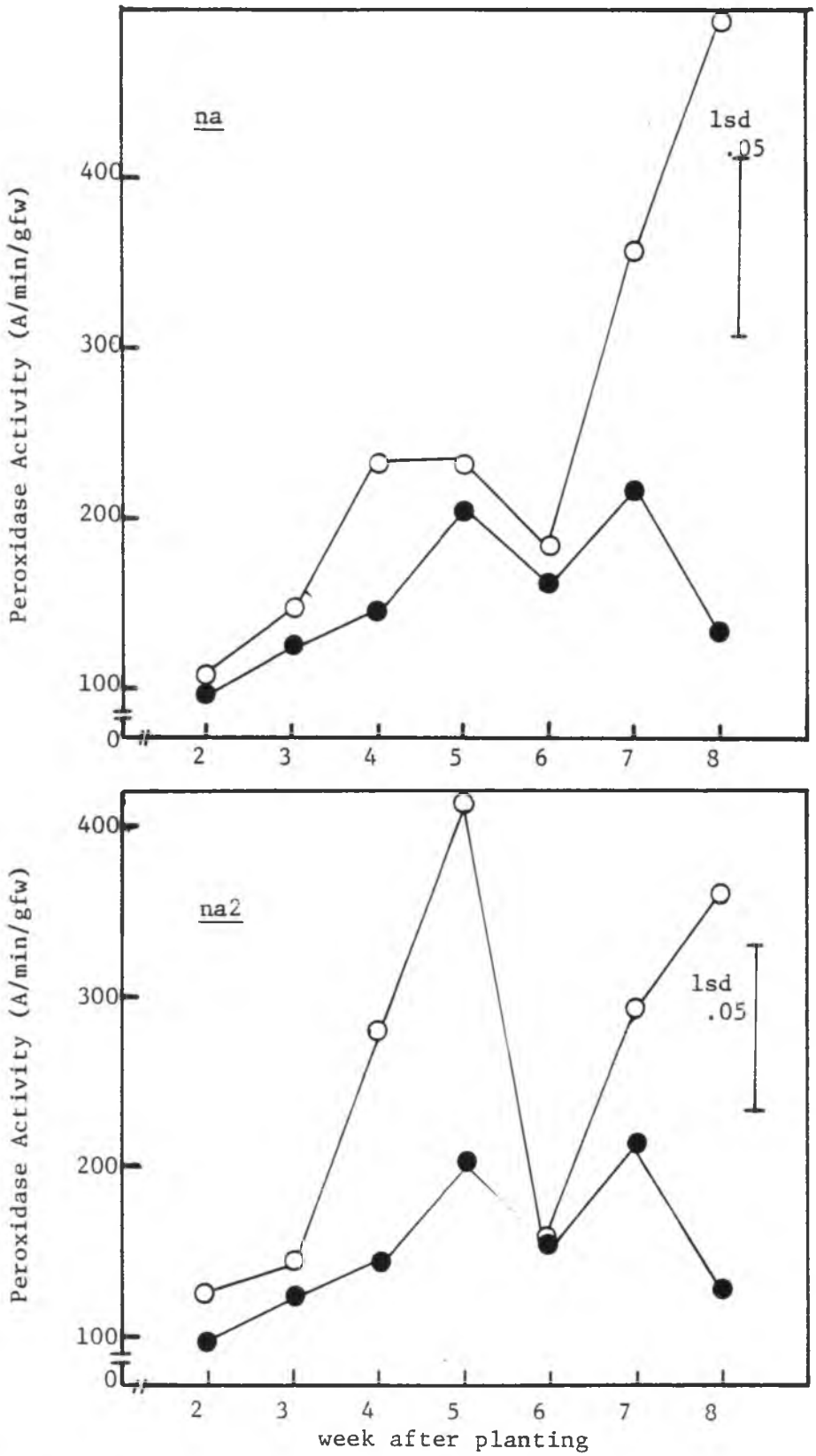


Figure 22. Peroxidase activity during development of dwarf mutants: (a) na, (b) na2 (o), and Hi27 check (●).

than twice the activity of control. Even though the phenotypic appearance of na2 was earlier than that of na, the peroxidase increase in both mutants was later than their phenotype appearances. The increased peroxidases at the later stage of growth appeared to be the result of altered metabolisms by na and na2 phenotypes.

The mutant py showed a rapid increase in peroxidase activity between 2 to 5 weeks of growth (Figure 23). During this period py started to display its shortened phenotype. The timing of growth retardance and peroxidase increase may imply the role of peroxidase in IAA degradation. Pygmy is one of the mutants reported as having low auxin in coleoptile (Overbeek 1938). Increased peroxidase in py may be related to auxin levels in the plants, but if it is the direct cause of shortness or not is not clear from this result. Like na, na2, py has very short leaves with a rough leaf surface. The increase in peroxidase activity may be related to this characteristic of py; however, no reports were found regarding difference in leaf components of py.

The cr mutant, which is dwarf plant and with semi-dwarf leaves, showed no significant difference in peroxidase activity (Figure 24). In this mutant the phenotype was mainly repressed in plant height dwarfness, thus leaf peroxidase may not be involved in this dwarfism.

Dwarf mutant peroxidases and Hi27 were examined each week for their isozyme pattern. Densitometric tracings from one week to the 7th week stage of Hi27 isozymes showed that two anodal peroxidases, Px8 and Px6, decreased, while Px3 and Px7 intensity increased. Cathodal isozymes Px4 and Px1 also decreased. No new peroxidase bands

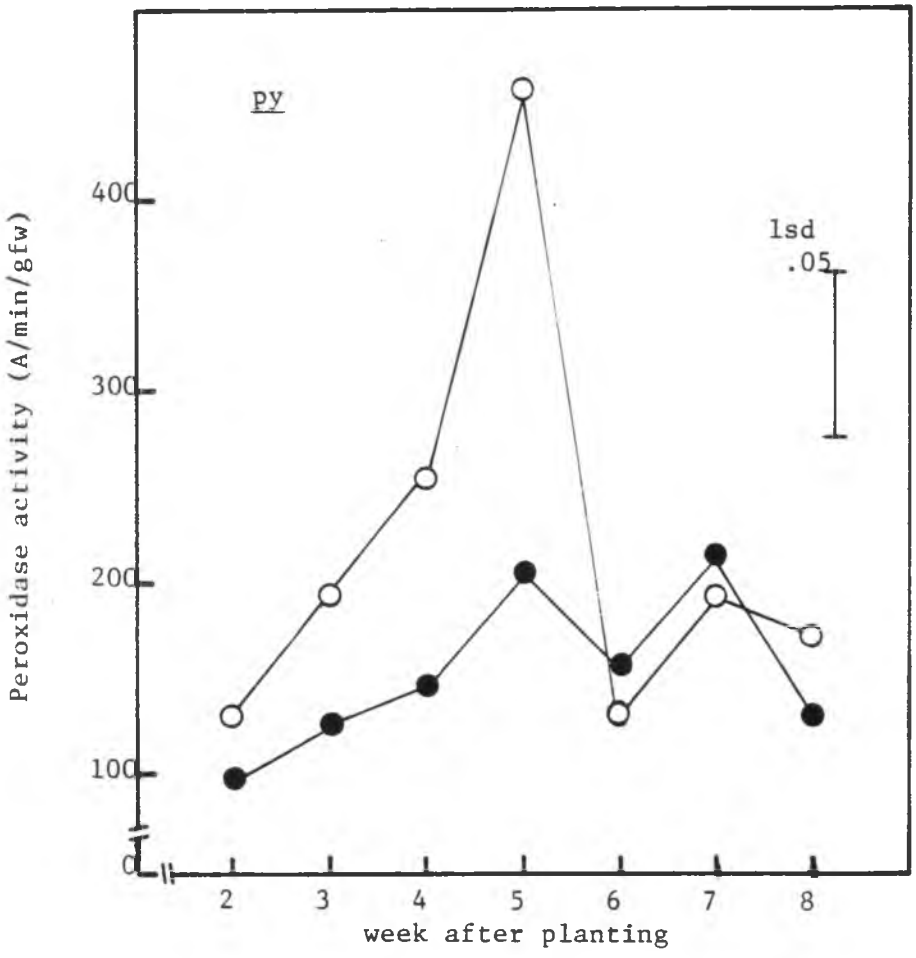


Figure 23. Peroxidase activity during development of a dwarf mutant: py (○), and Hi27 check (●).

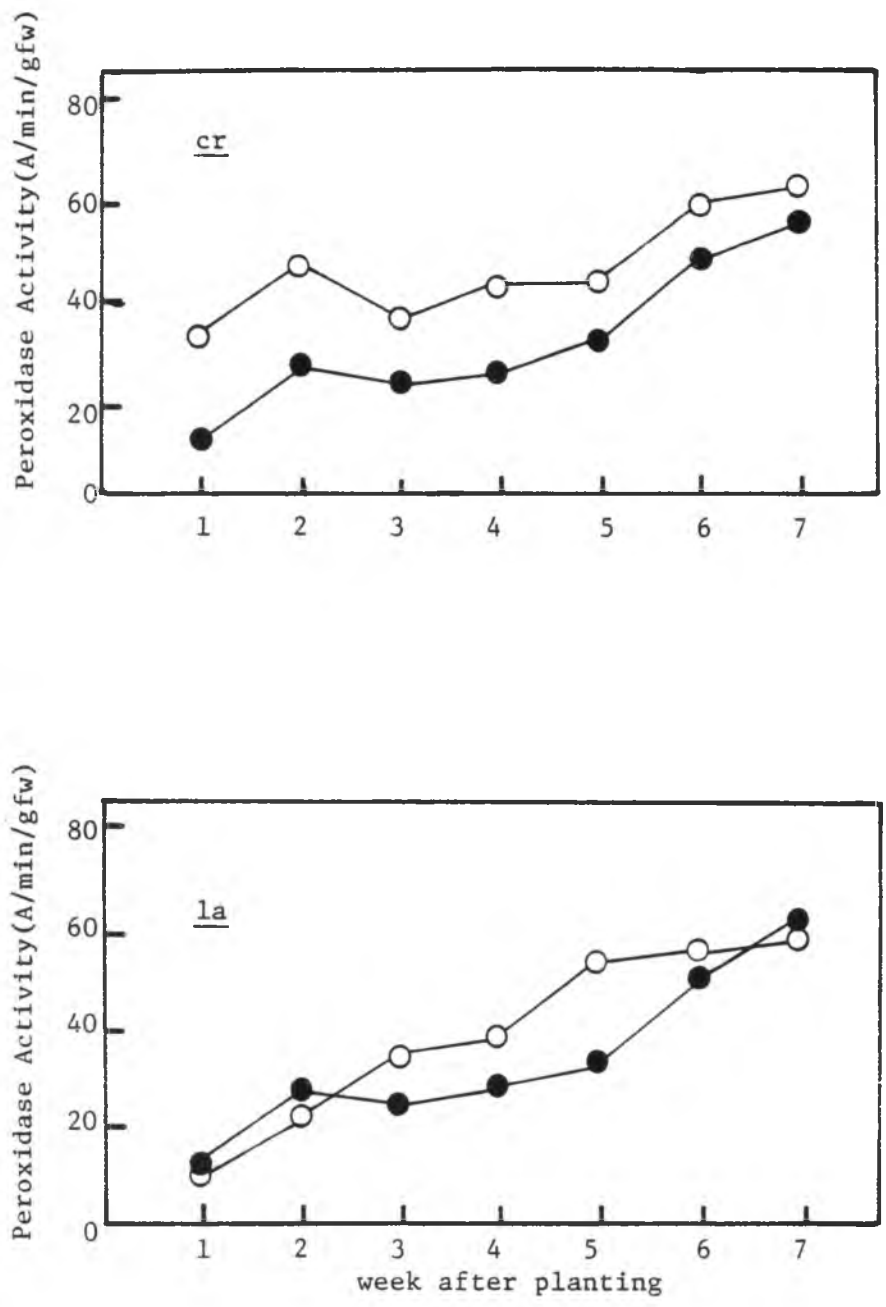


Figure 24. Peroxidase activity during development of mutant plants: (a) cr, (b) la (O), and Hi27 check (●).

appeared when the activity of the dwarf mutant peroxidase increased. From 1 to 3 weeks no change of peroxidase pattern was found between Hi27 and dwarf mutants. Figure 25 shows the isozyme pattern of dwarf mutant peroxidases at 4 week stage when activity started to change between Hi27 and mutants. Compared to equal intensity of Px3 and Px7 in Hi27, d, d-tn, br, br2, na, na2 and py showed higher intensity in Px7 than Px3. The na2 mutant showed the Px8 had which does not appear at this stage of normal leaves. This pattern did not change after the enzyme activity was equalized by dilution. After 5 weeks, no clear isozyme pattern difference was detected among mutants. Px8 was found in stele of mesocotyl (Brewbaker and Hasegawa 1975) and showed relatively higher activity with lignin precursor, eugenol and with IAA as did Px3 and Px7. Increased peroxidase of Px3, Px7 and Px8 in na, na2, br and py may function in IAA oxidation and lignification. The common phenotypes of 3 mutants na, na2 and py, which have stiff and pointed leaves, may be related to their peroxidase increase.

Among 8 dwarf mutants, na, na2, br, br2 and py increased in their peroxidase activity, while d and d-tn had peroxidase activities similar to Hi27. Two dwarfs, d and d-tn, were GA-responding type dwarfs (Phinney 1956), while na, na2, br and br2 were reported to have no response to GA. A study of timing between phenotype appearance and peroxidase activity increase showed two different groups. The first group includes br and br2 in which peroxidase activity increase and dwarf phenotype appeared at the same time (4-5 weeks). The other group of dwarfs are na, na2 and py. Their phenotype appearance was much

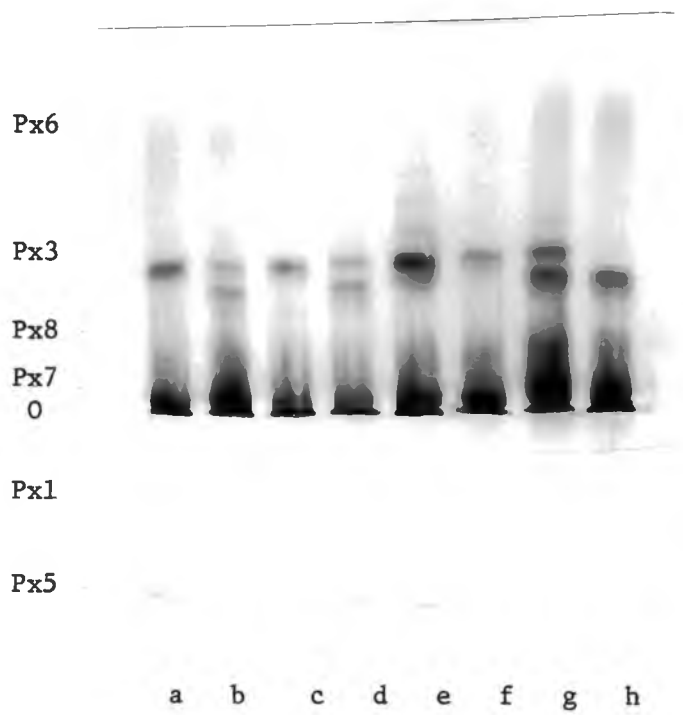


Figure 25. Zymogram of peroxidase isozymes from 4 week mutants and Hi27. (a) Hi27, (b)br, (c)br2,(d) d, (e) d-tn, (f)na, (g) na2, (h) py.

earlier than the stage of peroxidase elevation. High peroxidase activity in various dwarf plants has been attributed to the peroxidase role as an IAA oxidase (Birecka and Galston 1970). These authors suggested that a lowered level of IAA by peroxidase activity may cause shortness in plant height. However, the results obtained here indicate that only in two dwarf mutants (br and br2) can a similar peroxidase role be considered. In the second group mutants, na, na2 and py, there appeared to be no relationship between the timing of increased peroxidase activity in the leaf and phenotype appearance of the plant. Peroxidases of the leaves do not seem to play a direct role in dwarfing of these mutants, instead, they appear to be biochemical responses when plant growth is altered by mutant genes. Oxidation of phenolic compounds and IAA and lignin biosynthesis may be examples of this type of peroxidase function.

4.3.4 Peroxidase Activity in Other Types of Morphological Mutants.

Morphological mutants la, Kn, sl, Rg, gt and Cg (Table 13) were also examined. Kn had higher peroxidase activity after 2 weeks and it reached 14 times higher at 5 weeks than 1 week (Figure 26). Compared to the activity of Hi27, peroxidase activity was 4 times higher. Kn had tumorous tissues around the vein region of the leaves. Gelinas and Postlethwait (1969) reported that Kn/Kn leaves had higher IAA oxidase inhibitor than normal plants at the 14 day stage of plant growth. The amount of IAA oxidase inhibitor and total phenol content, however, decreased between 2 and 4 weeks in Kn/Kn plants. If the results of Gelinas and Postlethwait (1969) are applied to the present

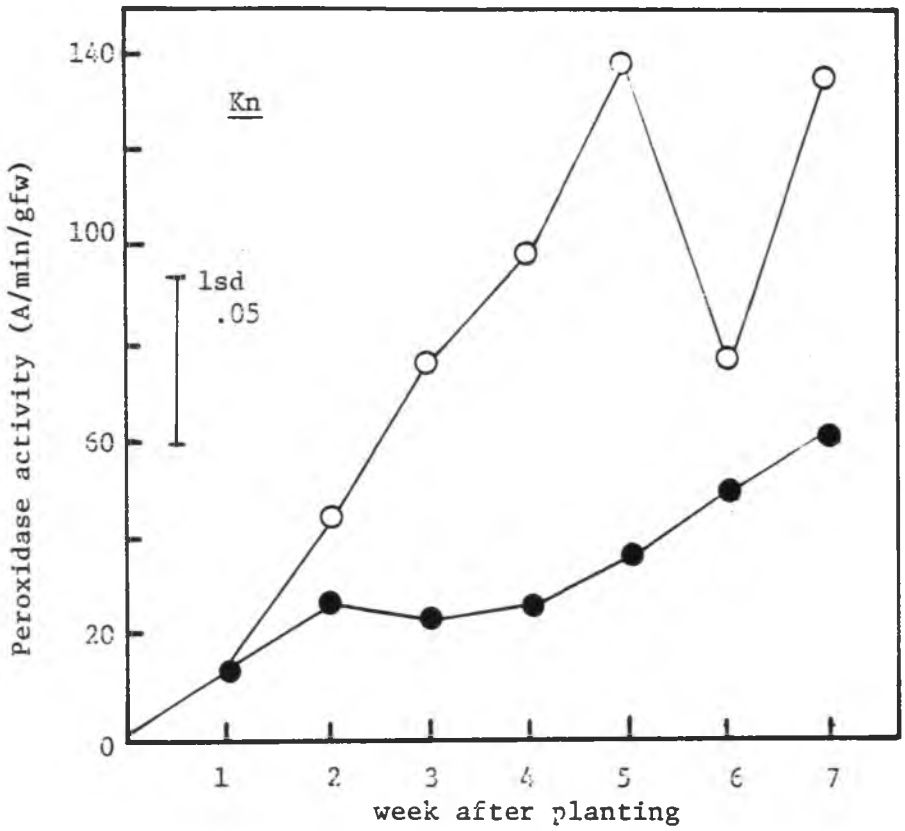


Figure 26. Peroxidase activity during development of heterozygous Knotted plants, Kn/+ (○), and Hi27 (●).

Table 15. Peroxidase activity in Knotted mutant (Kn) at 5 weeks.

Tissue	Total peroxidase activity	Specific activity
	— A/min/gfw —	— A/min/mg protein —
Leaf	45.92	11.90
Stem	48.00	18.26
Knotted part	66.29	18.36

study, the elevated peroxidase activity functions in phenol oxidation. The role of auxin has been recognized in the process of tumor formation in Nicotiana (Ahuia and Gupta 1974). They found that a specific peroxidase, A3, appeared after tumor formation in Nicotiana and suggested that high IAA in pre-tumorous plant repressed A3 locus.

In Kn, peroxidase activities in leaf, stem and tumor (knotted parts) were compared at the 4 week stage (Table 15). Peroxidase activity was higher in the tumor than in non-tumor leaf tissue. Specific activities in stem and tumor were not different. The isozyme pattern of the knotted part was the same as leaf peroxidases. The results indicate peroxidase involvement in knotted tissues related with IAA oxidation and phenol oxidation.

Mutant la which shows prostrate growth habit after two weeks of growth was examined for peroxidase activity (Fig. 24b). No significant differences were found through the stages. Peroxidases seem not to be related to the prostrate growth of la, which is a form of positive geotropism.

The grassy mutants, Cg and gt were examined for peroxidase activity. There were no significant differences found between leaf peroxidases of these mutants and normal plants. Mutant gt had similar leaf length and plant height as Hi27 (Table 14), which may account for the similar level of peroxidase activity. Cg was found to have a higher activity between 2 and 3 weeks of development in a preliminary study (Brewbaker and Park 1977), but this could not be verified.

4.3.5 Quantitative and Qualitative Changes of Peroxidases in Ragged Leaf (Rg) and Slashed Leaf (sl) Mutants.

Peroxidases were assayed quantitatively and isozymically in leaf tissues from Ragged leaf (Rg locus) and slashed (sl locus) mutants in stocks isogenic with inbred Hi27. Both Rg and sl have aborted leaf tissues with necrotic parts. Peroxidase activity was quantitized weekly for the first seven weeks of development until early flowering, using leaf samples as in previous studies. During this period the activity increased very slightly in the inbred Hi27 check compared to each of these mutants (Figure 27), in which specific activity of peroxidases was elevated about 2-fold for sl and 8-fold for Rg. Details of these responses and the developmental morphology of these mutants are described in the following sections.

Rg: The Ragged leaf mutant (available only as heterozygote Rg/+) has short and necrotic leaves and dwarf plant stature. This severe phenotype often causes poor seed formation, and homozygotes are normally sterile. Dwarfing in Rg plants was very severe and leaf length was only 30% of that of control. The phenotype appeared as early as the 3rd week stage (Table 13). The peroxidase activity in Rg mutant increased rapidly in the late stage of development between 5 and 7 weeks (Figure 27). At 6 and 7 week stage it reached to 8 times higher than control. Specific activity increase was observed similar to that of total activity (Figure 28).

Plant height of Rg plants is shown in Figure 28. Between the 4 and 6 week stage, retardation of Rg plants became more severe than in earlier stages. The increase in peroxidase activity started 2 weeks

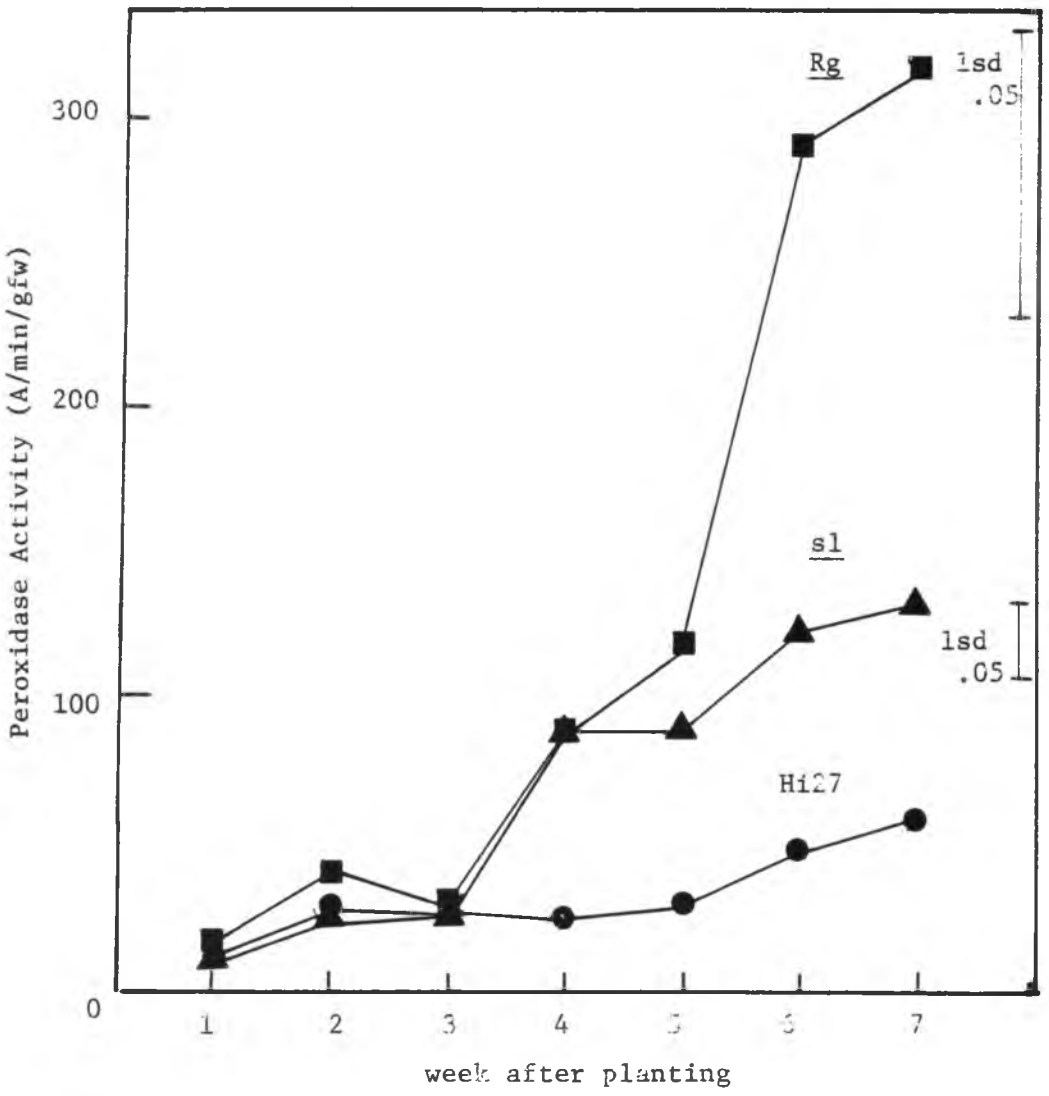


Figure 27. Peroxidase activity on gm fresh weight basis during development of mutant maize plants, Rg (■), sl (▲), and Hi27 check (●).

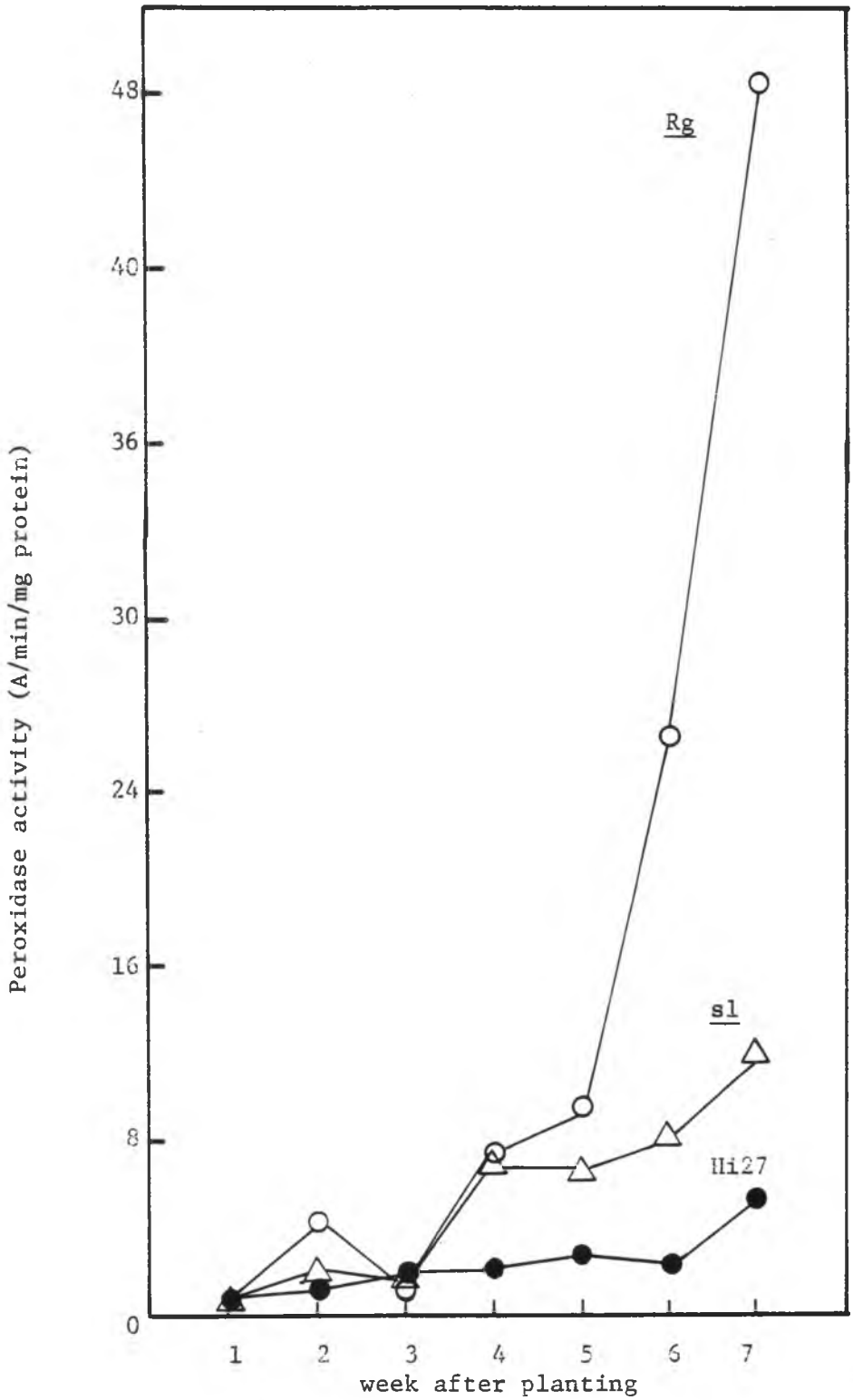


Figure 28. Peroxidase activity on mg protein basis during development of mutant maize plant: Rg(○), sl(△) and Hi27(●).

later than Rg phenotype appearance. Apparently peroxidase activity is not solely related to growth rates, but rather may play a physiological role in phenol oxidation or lignin synthesis which indirectly control growth rates. Necrotic parts in leaves of Rg seem to be similar to fungus infected tissues (Birecka and Garraway 1978, Vance et al. 1976); e.g. H. maydis, in which high lignin formation and peroxidase activity were observed. Involvement of ethylene in these tissues is probable. If the 'unhealthy' condition of leaf tissues caused by Rg is similar to stress condition caused by wounding or disease, then the high peroxidase level in Rg leaf may be derived from wound-generated ethylene.

S1: The slashed mutant showed elevated peroxidase activity in the fourth week, maintaining thereafter an activity about double that of control (Figure 27, Figure 28). The mutant is not evident morphologically in young seedlings, but by the fourth week the small slashes appear parallel to veins in the leaves as evidence of tissue breakdown in localized regions of the leaf. The slashed plants show a slight dwarfism, which is also first evident around the fifth week (Figure 29). Thereafter the slashed plants remain about 25% shorter than normal. After the 4th week of development, peroxidase activity in s1 maintained the level significantly higher than that of control (Figure 27). Both total activity and specific activity (Figure 28) of s1 were twice as high as Hi27. Peroxidase activity increase started at the same time that s1 plant growth was retarded. This timing of peroxidase increase implies a possible role of peroxidase of

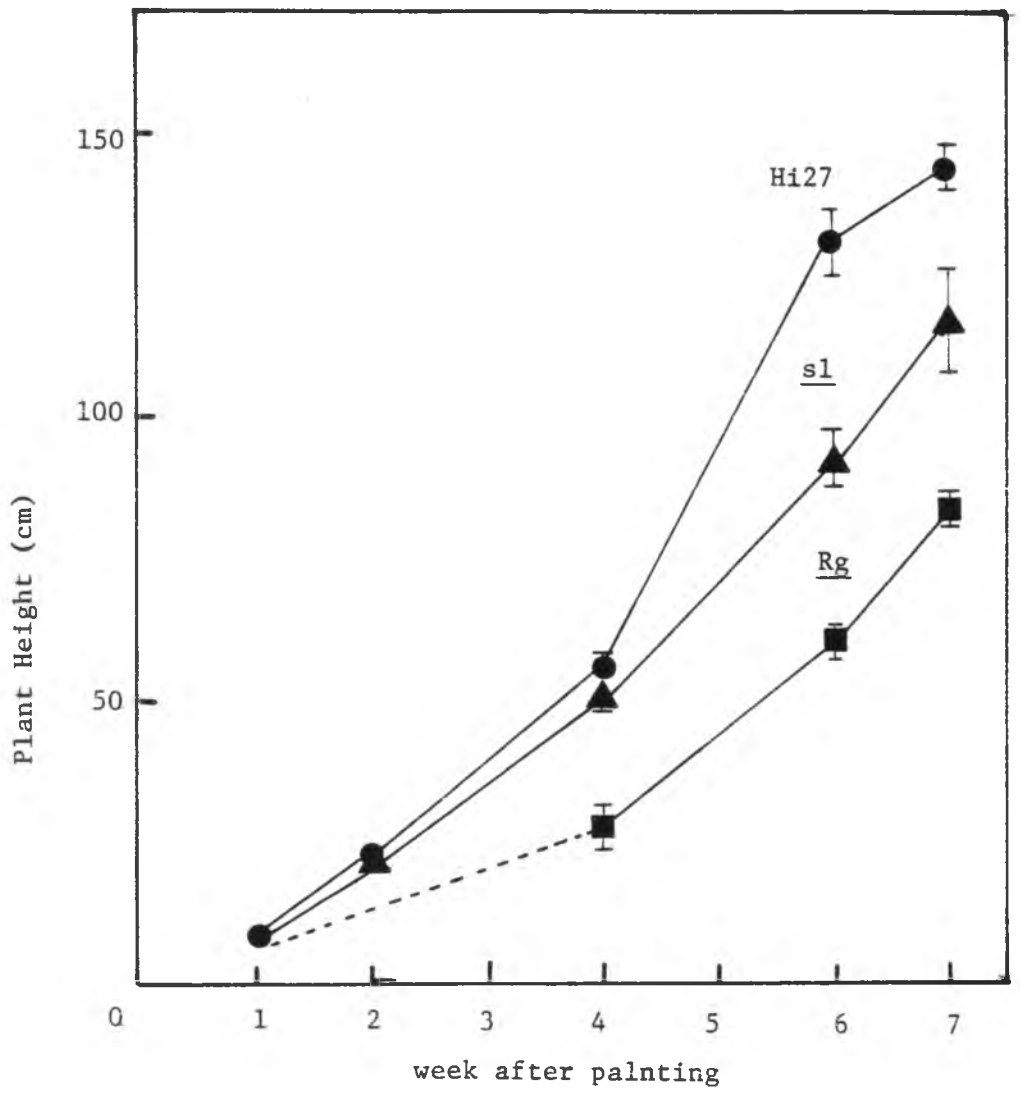


Figure 29. Change of plant height during development of mutant maize plant Rg (■), sl (▲) and Hi27 check (●).

sl in IAA degradation. Higher level of peroxidases between 4 and 7 weeks may keep sl plant height shorter than control.

Substrate specificity of peroxidases in leaves of 6 week old plants was examined to investigate the qualitative differences in peroxidases of mutants Rg and sl (Table 16). Specific activity was compared on o-dianisidine, benzidine and guaiacol substrates. In Rg mutants the activity of peroxidases on guaiacol and benzidine substrates was 1.5 times higher than that of Hi27. Substrates o-dianisidine and benzidine are artificial dyes, while guaiacol is one of the products of pyrolyzed lignin (Siegel 1957). High substrate specificity of Rg to guaiacol may indicate that increased peroxidase activity in Rg is involved in lignin formation. The result of the substrate specificity study also indicates that Rg may have dissimilar isozymes compared to Hi27, at this stage.

Qualitative differences in peroxidase isozymes at the 5th week stage were examined using electrophoresis. Zymograms of peroxidases from 5 week leaves are shown in Figure 30. The Hi27 zymogram showed isozymes Px6, Px3 and Px7 in the anodal region (Figure 30). When Rg peroxidase was examined, however, a wide band just below the Px3 region and in addition to Px6 and Px7 was identified (Figure 30). This newly appeared band was designated as Px-Rg. In sl a very weak band of this unusual band was also observed (Figure 30). Band intensity of Px6 and Px7 was not different from Hi27 and Rg, while Px3 was higher in Rg than in Hi27. The high peroxidase activity noted in Rg appears to be associated with Px3 and the newly identified Px band (Px-Rg).

Table 16. Substrate specificity of peroxidases from 5 week normal and mutant maize leaves.

Line	Activity Means and Standard Errors on Substrates: o-Dianisidine		Guaiacol		Benzidine	
	ΔA/min/mg protein (percentage[1])					
Hi27	4.22 ± 0.19	(100)	0.42 ± 0.16	(100)	3.97 ± 0.80	(100)
sl	7.28 ± 0.76	(173)	0.83 ± 0.61	(197)	6.42 ± 0.19	(162)
Rg	9.34 ± 1.88	(222)	1.45 ± 0.23	(345)	14.80 ± 1.10	(372)

1) Expressed as Hi27 = 100%.

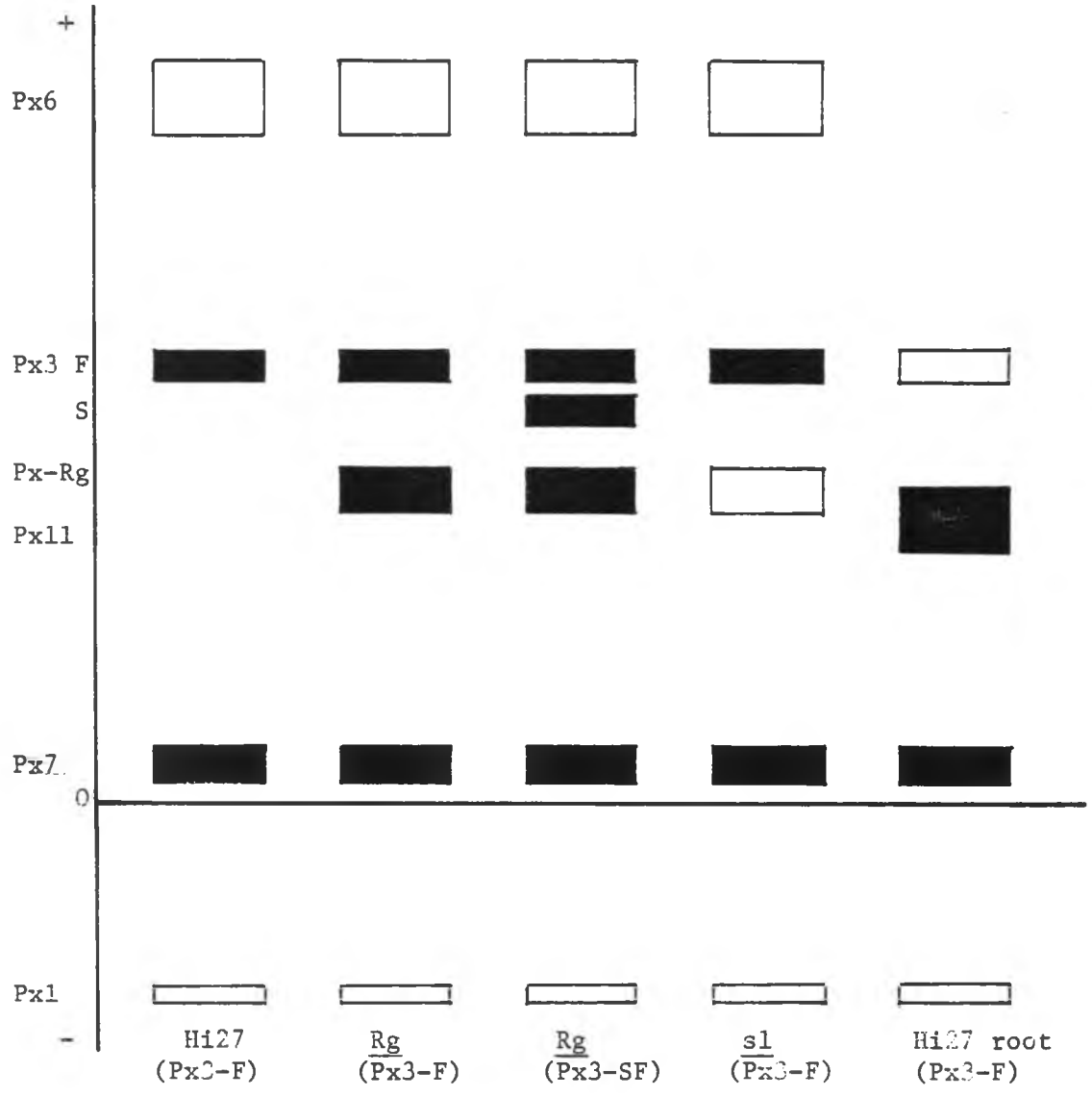


Figure 30. Zymogram of peroxidase isozymes of 5 week leaves of Hi27, Rg (Px3-F), Rg (Px3-SF) and sl compared to root tissue of Hi27.

Zymograms of Rg plants of two genotypes of Px3, Rg (Px3-S/F) and Rg (Px3-F), were compared in order to determine the origin of the new peroxidase band. In both genotypes of Px3 in Rg (Figure 30), the new band was located at the same position on the zymogram. Since the band location was the same in both genotypes, Rg (Px3-F) and Rg (Px3-S/F), the new band seems not to be influenced by the genotype of Px3. Densitometry record showed the new band location more clearly (Figure 31). In the Px3 region, multiple bands of decreasing intensity away from origin have been observed in old tissues (Peirce and Brewbaker 1973) and rust infected leaves (Kim et al. 1978). The Px-Rg band observed here, however, was different in location and width from the multiple bands of Px3. From the evidence reported here, Px-Rg appears to be distinct from Px3, but additional testing is required to verify it. Some possibility remains that the new band, Px-Rg, is a derivative of Px3 due to an alteration of an enzyme conformation such as a sugar moiety change.

Px11 is another peroxidase isozyme, which is located in the anodal region close to the new band, Px-Rg. Px 11 exists in root, brace root tissues and callus from apex and endosperm in maize (Brewbaker and Hasegawa 1975). The location of the new band and Px11 was compared using root extract of maize and electrophoretic methods (Figure 30). The wide band of Px11 was located at a slightly lower zymogram region than the new band. Repeated electrophoresis confirmed by densitometry a slight mobility difference between the wide band of Px11 and the new band (Figure 31). The gel stain of Px11 showed relative activities with benzidine, o-dianisidine, eugenol and

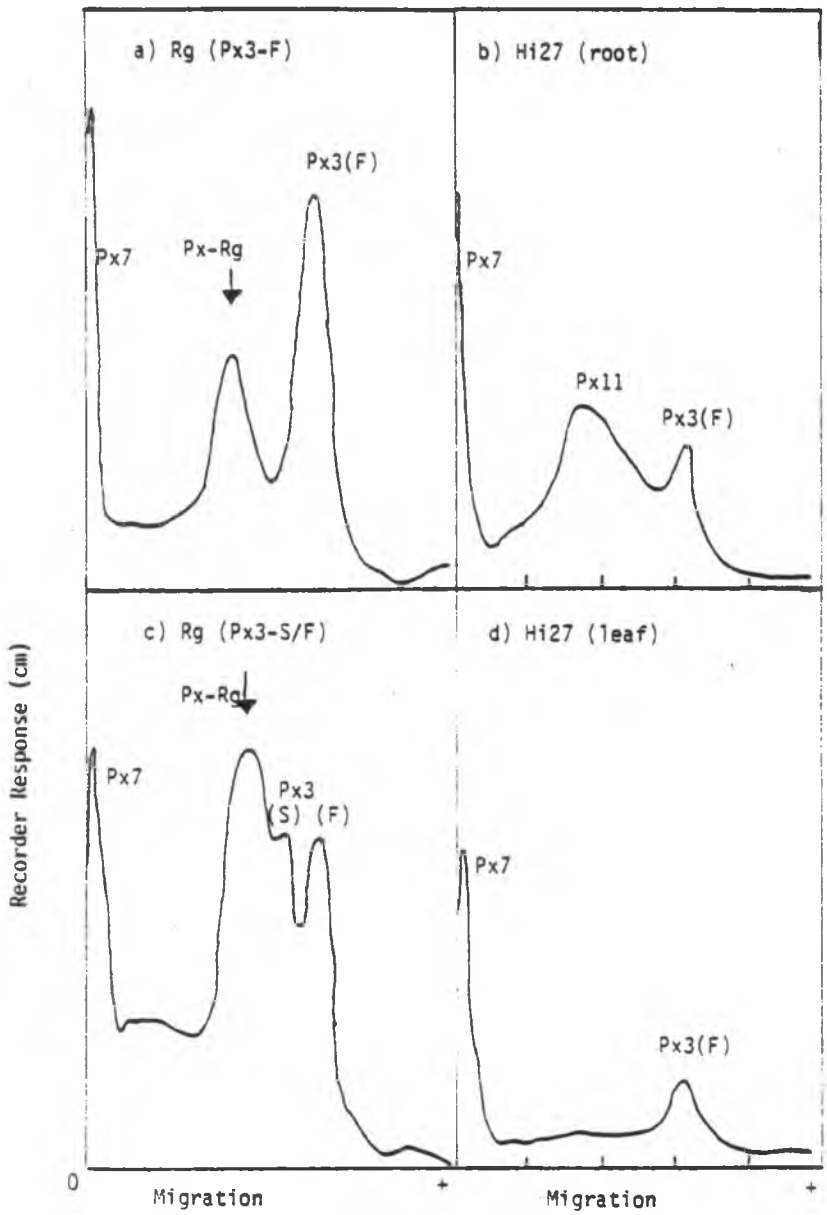


Figure 31. Densitometric trace of peroxidase zymograms for 5 week old leaves of a) Rg (Px3-F), b) Rg (Px3-SF), c) Hi27 (Px3-F) compared to Hi27 roots (Px3-F).

indole acetic acid similar to those of Px3 and Px7 (Brewbaker and Hasegawa 1975). These authors also found that Px11 had lower activities with pyrogallol and guaiacol when compared with Px3 and Px7. Higher specific activity of Rg peroxidases to guaiacol, however, does not correspond with Px11 having a lower activity with guaiacol. This indicates that the new band has different substrate specificity from Px11. However, results from the gel stain of Brewbaker and Hasegawa (1975) and the spectrophotometric method used here, might have caused this difference. Yet, Px11 is still the closest peroxidase isozyme to the region and there is a strong possibility that the new band is a modified Px11 existing in leaf tissue.

If the new band Px-Rg, is a modified band of Px11 of roots, the Px11 gene may be derepressed in Rg leaf tissues whereas under normal condition Px11 is absent. The direct trigger which derepressed the Px11 gene may not be the Rg mutant gene. Appearance of the new band, Px-Rg, started at the 5th week stage development, while the phenotype of Rg was already detected at 3 week stage. The trigger might be the altered hormonal balance or substances caused by the biochemically altered tissues of Rg. Induction and repression of specific peroxidase isozymes by growth regulators such as IAA, GA and ethylene have been observed in various plants (Galston et al. 1968, Dendsay and Sacher 1978). Further investigation using these growth regulators may solve the question whether the new band in Rg is due to derepression of Px11 gene or not. Peroxidase study in morphological mutants by many researchers showed no qualitiative

difference in electrophoretic pattern (Price and Stebbins 1971, Schertz et al. 1971, Evans and Alldridge 1965). The results presented here are important evidence that increased peroxidase activity in mutant maize plants is followed by the appearance of additional bands.

From the experiments presented here, no evidence of specific physiological role of the peroxidase isozyme was found. Extensive substrate specificity studies might provide information on the affinity of this new band to natural substrates such as lignin precursors. This approach could clarify the involvement of this newly identified peroxidase in lignin biosynthesis and phenolic oxidation.

4.4 Wind Effects on Maize Peroxidases

Wind has been found to decrease the growth and yield rates in many crops. Reduction of plant height, damage to tissue surfaces (MacKerron 1976), increase in resppiration rates (Todd et al. 1972) and reduction of photosynthetic rates (Grace and Thompson 1973) have all been attributed to the effects of wind. In Hawaii, reduced plant height and yield in maize have been observed in various windy locations, e.g. Kohala on the Island of Hawaii. Peroxidase activity increases and growth decreases have also been observed in plants exposed to mechanical perturbations (Boyer and Chapelle 1979), such as might be expected from wind stresses. In an experiment with a mutant series conducted at Waimanalo, background peroxidase activity (Hi27) was higher and more variable in winter months than during spring months. At 7 week Hi27 plant growth peroxidase activities of the 3rd leaf were 210 A/min/gfw and 60 A/min/gfw for winter (October planting) and spring (April planting), respectively. Environmental factors which cause these differences in peroxidase activity may include continuous strong tradewinds during the winter season, althoug leaf diseases were also more severe in winter.

Maize leaf peroxidases from Hi27 and slashed mutants (sl) in field-grown and greenhouse-grown samples were compared to determine outdoor effects on peroxidase activity. Greenhouse grown plants were planted on the same day as field plants and measurements were made at two week stage plant height (10 plants) and the 3rd leaf peroxidase activity (3 plants) were measured. Bσth in Hi27 and sl, greenouse plants were taller than field grown plants (Figure 32a). At the 2nd

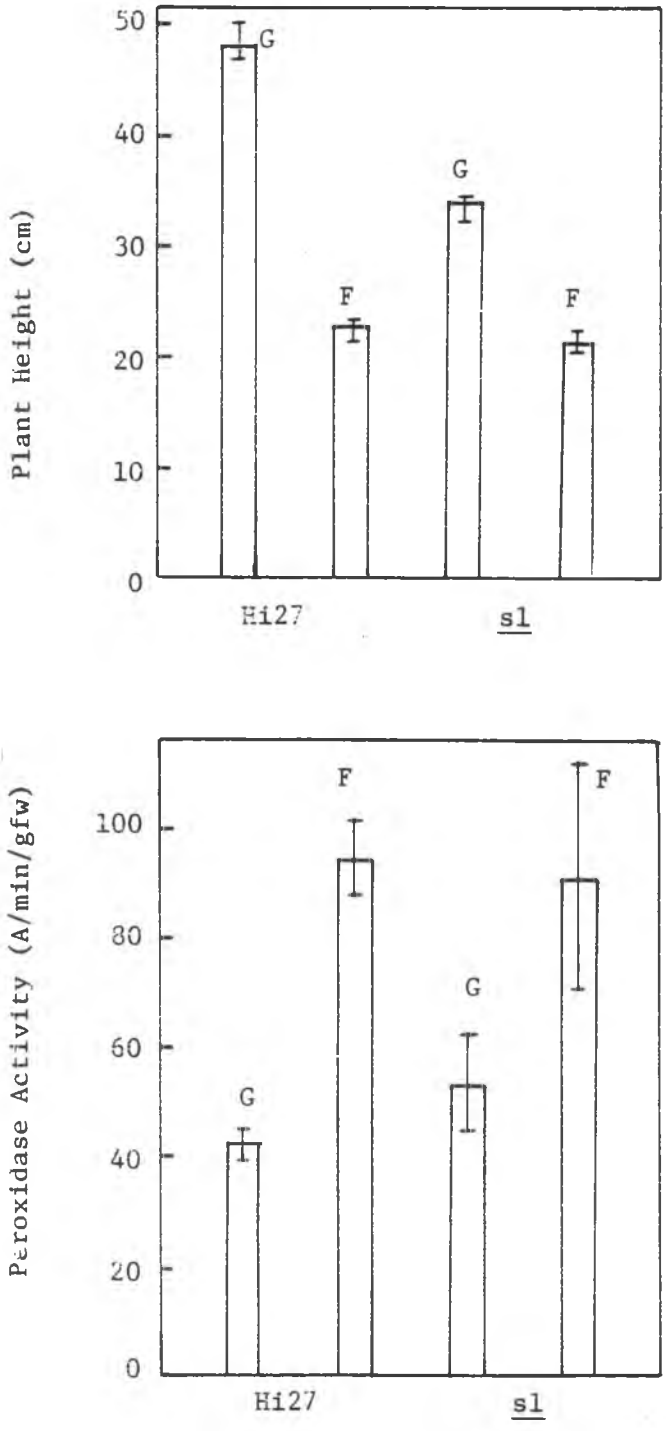


Figure 32. Plant height (a) and peroxidase activity (b) in the greenhouse (G) and field-grown (F) maize.

week stage, no significant difference was found between Hi27 and sl in field condition, however, in greenhouse Hi27 were significantly taller. In Hi27, peroxidase activity in field grown plants of Hi27 was twice that of greenhouse grown plants (Figure 32b). In sl, peroxidase activity in field grown plants was also higher. Variations of height and peroxidase within variety and place were greater in the field.

Several environmental factors may be responsible for higher peroxidase activity and shorter plant heights under field conditions. First, day temperature was different between greenhouse and field condition (average ± 8°C). Temperature may be a factor causing plants to grow faster in the greenhouse. Light intensity is another factor, however, no reports of temperature and light effects on peroxidase were found (except for cold hardiness). Another possible cause of peroxidase activity difference is rust infection of plants in the field. However, most of the young plants at 2 weeks of growth were not infected and the rust did not become apparent until 4 to 5 weeks when it appeared in the older leaves. Wind may be one of the environmental factors which changes peroxidase activity.

Wind stress experiments were conducted under controlled conditions in the greenhouse to examine whether wind altered growth rate and peroxidase levels in maize palnts. As a preliminary experiment to determine the wind velocity for treatment, two wind velocities, 1.8 m/s and 3.2 m/s, were used and peroxidase activity and plant height of 3 plants per treatment were measured at 3, 7 and 14 days after treamtnet was initiated. Plant height in wind treated plants was

higher than control at the 3 day stage, however, after 7 and 14 days there was no significant difference (Figure 33a).

No differences were found between control and 1.8 m/s wind treatment in peroxidase activity (Figure 33b). However, plants treated by 3.2 m/s wind showed higher activity than control at the 7 day stage. At 14 days, peroxidase activity in the three treatments was similar. Two reasons for these results are suggested. First, the variation of plants within the treatment was too large to detect differences between treatments and second, the wind velocities, 1.8 m/s and 3.2 m/s, were too low to affect detectable changes on plant metabolisms.

The results of this preliminary experiment were considered in the design of the second experiment. To reduce variation within a treatment, the number of plants harvested from each treatment was increased to 12. Two increased wind velocities; 3.2 m/s (Velocity I) and 4.2 m/s (Velocity II) were used. Detailed methods are as described in Section 3.7.

Wind treated plants showed a reduction in plant height 3 days after treatments were initiated (Figure 34). Plant heights of Velocity II (4.2 m/s) were markedly decreased between 3 days and 14 days, and heights in the Veolocity I (3.2 m/s) were intermediate between control and Velocity II. The Velocity II winds, comparable to Hawaii's tradewinds, significantly reduced the plant height of young maize. The results here correspond to reports of wind stress on tobacco (Akimoto et al. 1975) and ryegrass, Lolium pernne (Russel and Grace 1978).

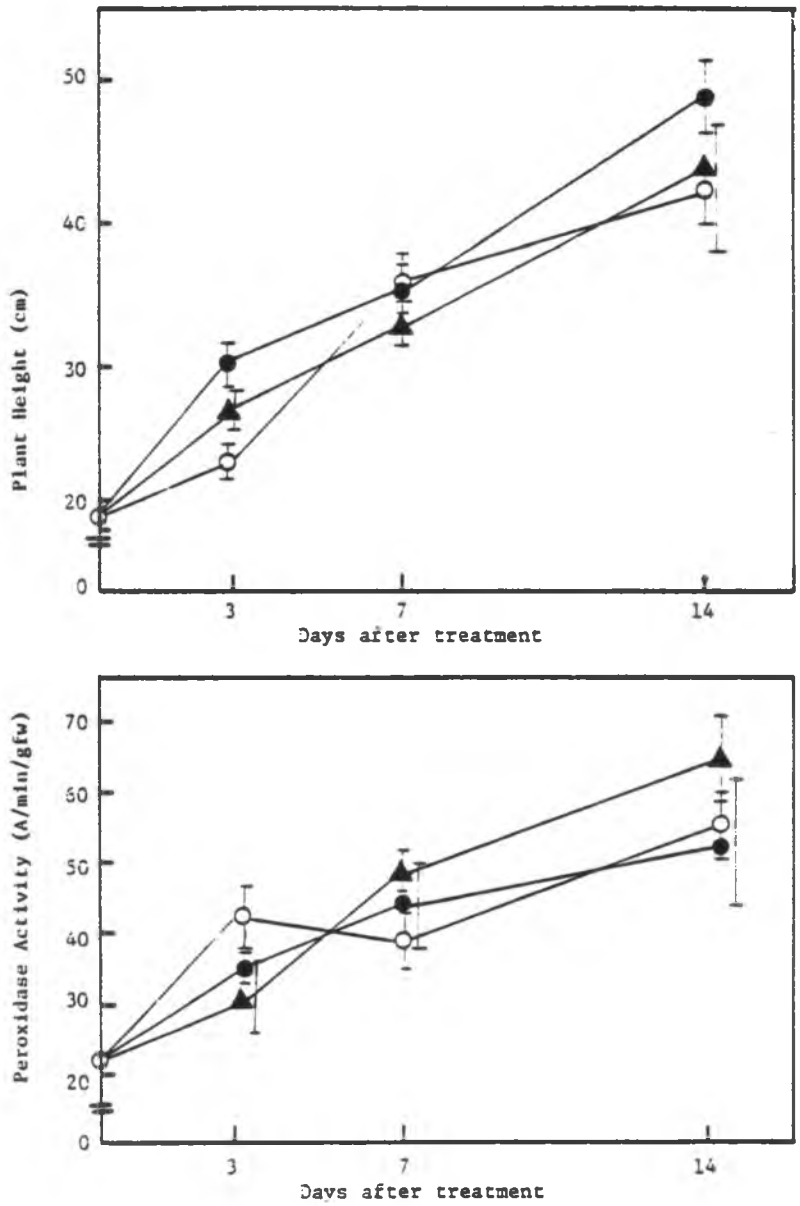


Figure 33. Plant height (a) and peroxidase activity (b) of wind treated plants. Wind velocity 1.3m/s (●—●), 3.2m/s (▲—▲) and control (○—○).

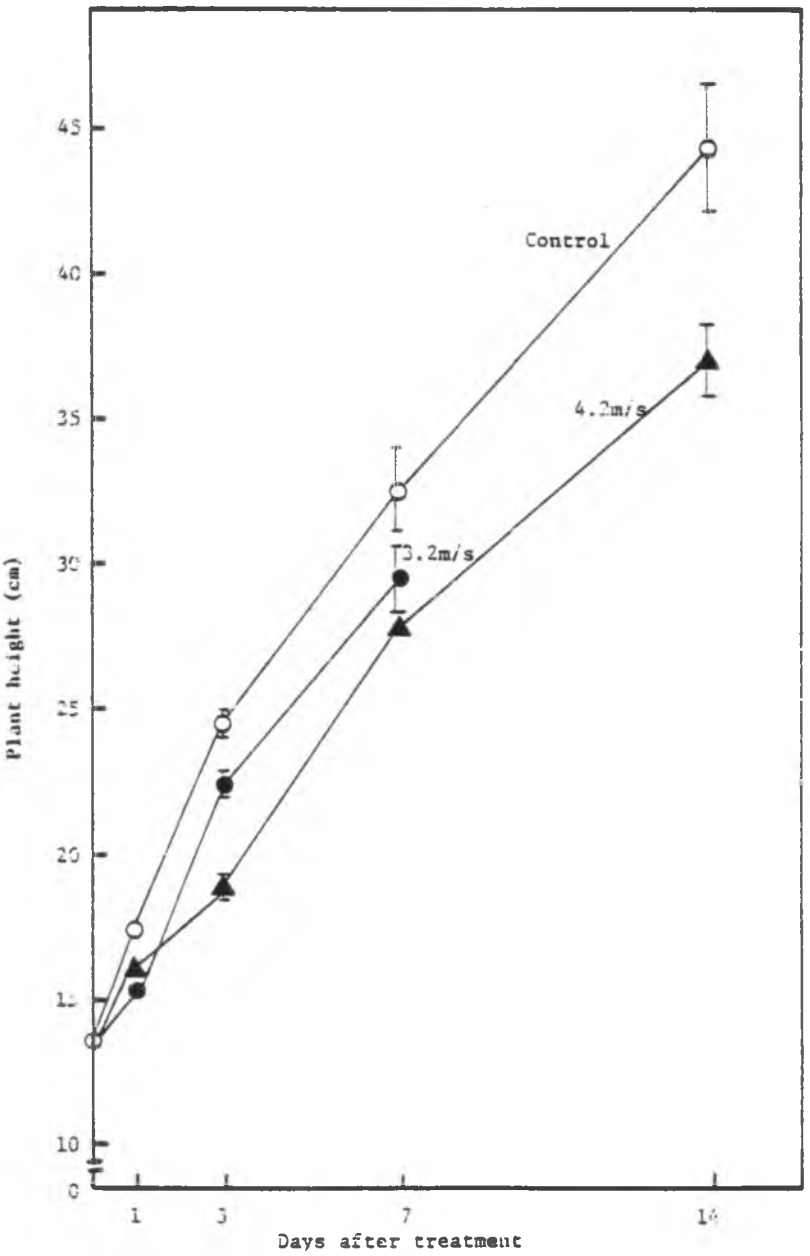


Figure 34. Plant height of wind treated maize plants. Wind velocity 3.2m/s (●), 4.2m/s (△), and control (○).

Fresh weight and dry weight of the upper portion of wind treated plants were compared (Figure 35). A difference in fresh weight between control and Velocity II was significant ($p < .05$) after 3 days (Figure 35a). At the 7 and 14 day stage, a significant increase of fresh weight of control was significantly greater than Veolocity II but not Velocity I. The dry weight of plants from Velocity I, between the 3 and 7 day stage, was the highest and those of Velocity II were the lowest (Figure 35b). A significant difference was found between the two levels of wind velocity treatment but not with control (Figure 35b). Todd et al. (1972) reported an increase in respiration rate in various plants, including maize, at wind velocity of 3.6 m/s and above. Wind Velocity I (3.2 m/s) may be a mild stimulating factor for plant growth in the greenhouse because the low velocity wind may cause air mixing which helps in replenishing the CO_2 supply for photosynthesis. Velocity II appears to be beyond the wind velocity favorable for plant growth. Accumulation of cell wall components by higher peroxidase activity in Velocity II may be another reason for no signfiicance between control and Velocity II.

Differences in peroxidase activity became detectable at the 3 day growth stage (Figure 36). Control plants showed similar peroxidase levels throughout the 14 days stage, while peroxidases from Velocity I and Velocity II plants showed increases in activity between 3 days and 14 days of treatment (no data for Velocity I at 14 days). Seven days after treatment there were significant differences between the peroxidase level of wind II plants and control plants (Figure 36). However, wind I plants and control did not exhibit significant

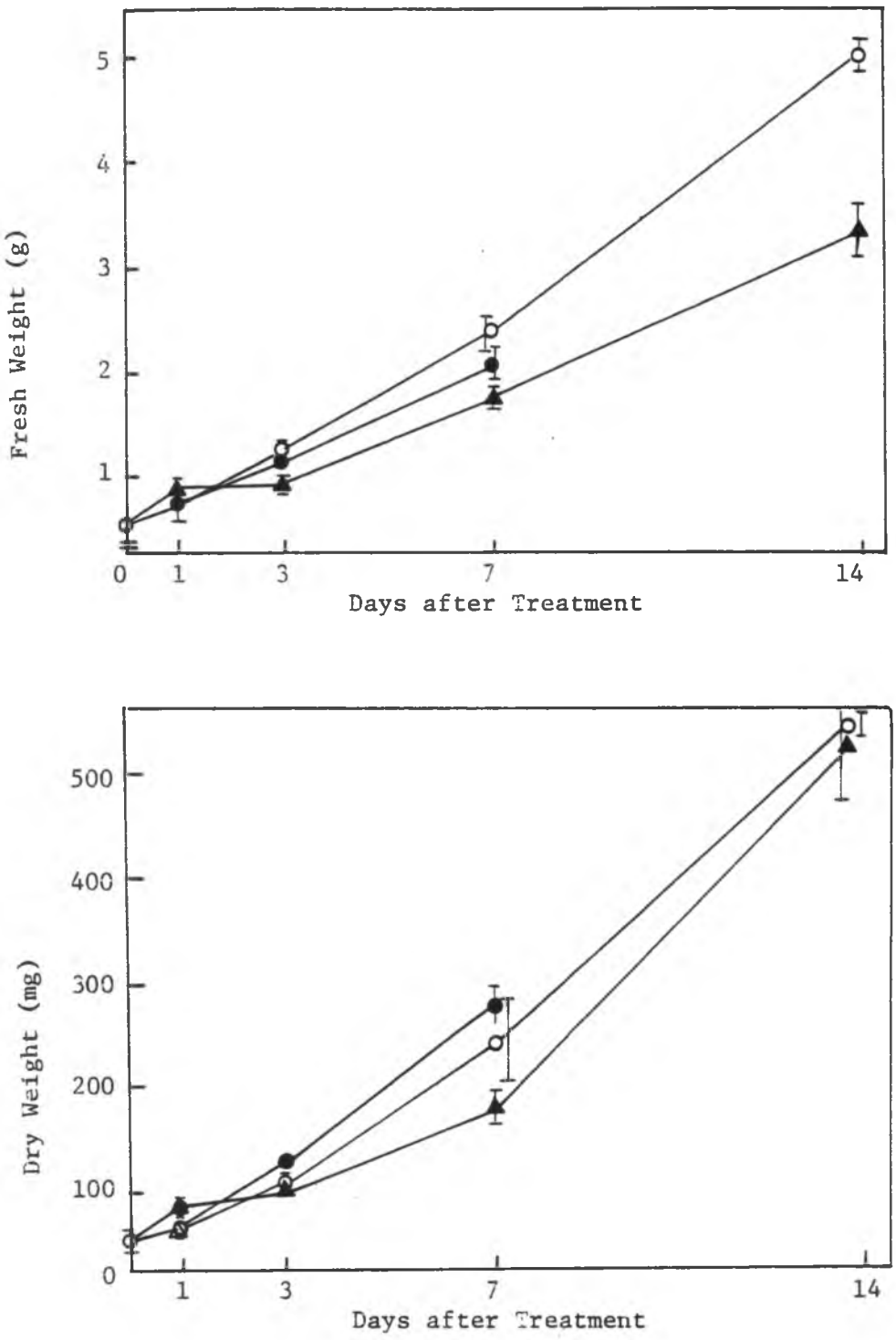


Figure 35. Fresh weight (a) and dry weight (b) of wind treated maize plants. Wind velocity I. 3.2m/s (●—●), II. 4.2m/s(▲—▲), and control(○—○).

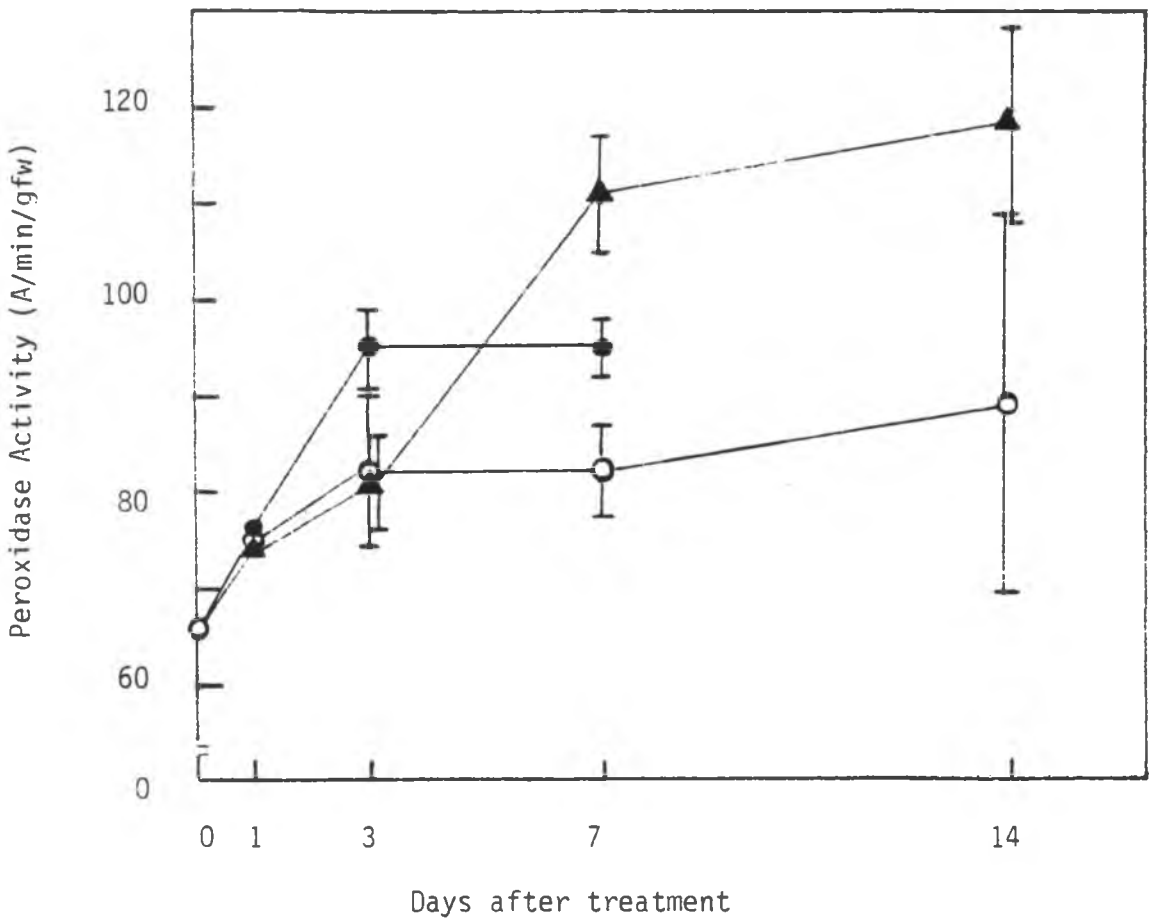


Figure 36. Peroxidase activity in wind treated maize plants. Wind velocity, 3.2 m/s (●—●), 4.2m/s (▲—▲) and control (○—○).

differences. At the 14 day stage, the average peroxidase activity of Velocity II plants was higher than that of control, however, the variation within a treatment was too large to be significant. Total activity and specific activity showed similar trends (Figure 36, Figure 37).

The results of 7 day stage, when the difference between control and wind treatment became clear, are shown in Table 17. Plant height and fresh weight of Velocity II plants were significantly lower than control, while peroxidase activity of Velocity II plants was significantly higher than control and Velocity I plants (Table 17).

At the 7 day stage, multiple bands in the Px3 region were observed in three out of five Velocity II treated plants. There have also been observed in old tissues and rust infected maize (Kim et al. 1978). No multiple bands were found in the control plants. The band pattern of other peroxidase isozymes exhibited no variation between treatments and control. Appearance of multiple bands appears to be caused by wind treatment.

As described earlier, the retardation of growth became significant at the 3 day stage, while increasing peroxidase activity was not observed until 7 days after treatment (Table 17). These results indicate the peroxidases increased after the initiation of growth retardation. Peroxidase increase appears not to be a preliminary response of wind stress in maize but rather a secondary response to physiological change. A similar relationship between peroxidase activity and plant grwoth was observed by mechanical rubbing of internodes of wild cucumber, Bryonia, internodes (Boyer

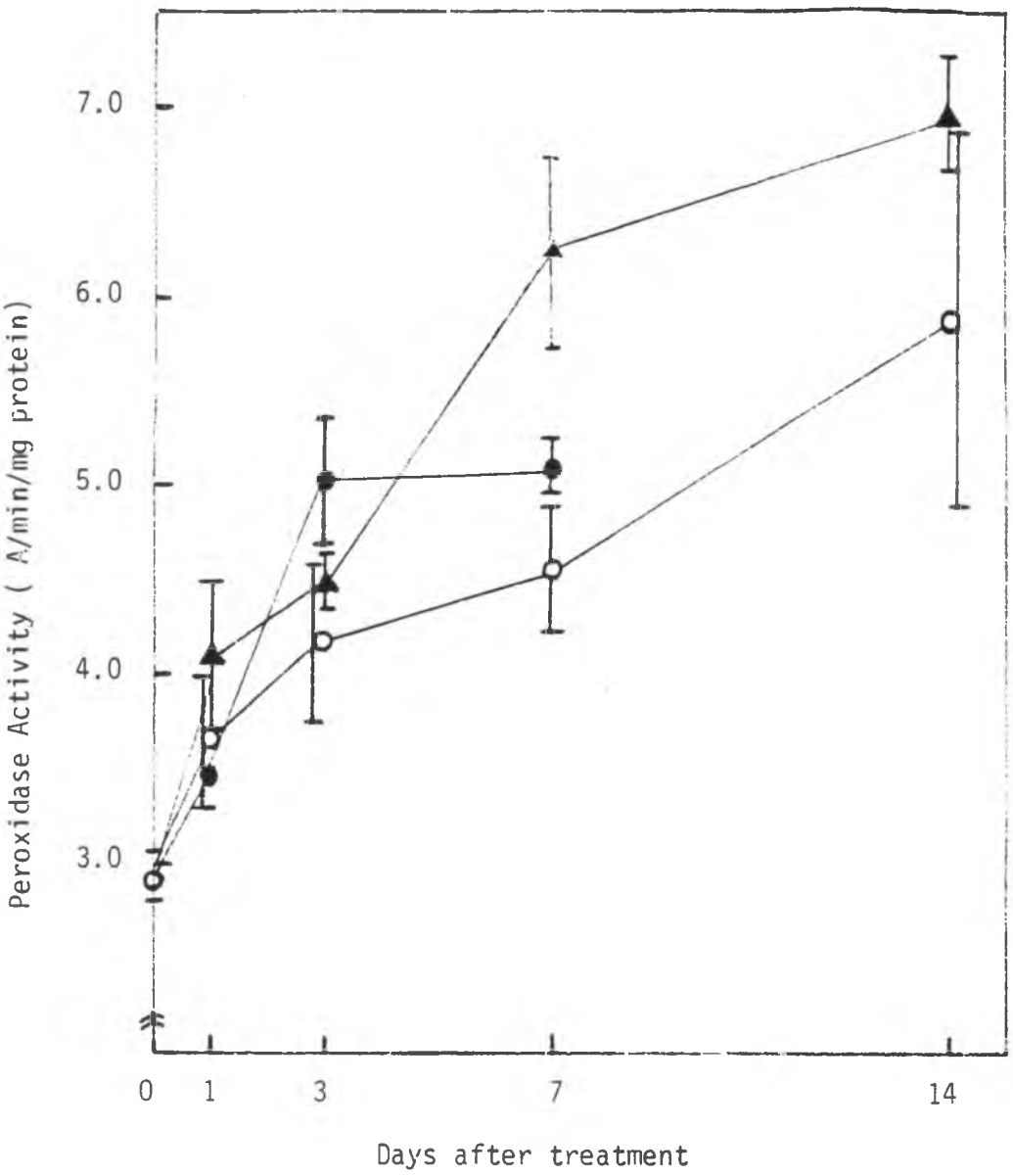


Figure 37. Specific activity of peroxidases in wind treated,10 day old maize plants. Wind velocity I 3.2m/s (●—●), II 4.2m/s (▲—▲) and control (○—○).

Table 17. Effect of wind on growth and peroxidase activity of maize.

Wind	Plant Height	Fresh Weight	Dry Weight	Total Peroxidase Activity	Specific Peroxidase Activity
	------cm------	------g------	------mg------	—A/min/gfw--	A/min/mg protein
3 days after treatment:					
Control	24.50 ± 0.43 b	1.28 ± 0.10 b	115.2 ± 10.80 a	82.00 ± 8.32 a	4.18 ± 0.43 a
Wind I -3.2 m/s	22.49 ± 0.36 b	1.26 ± 0.07 b	132.2 ± 11.60 a	95.21 ± 4.44 a	5.12 ± 0.31 a
Wind II -4.2 m/s	18.73 ± 0.51 a	0.98 ± 0.06 a	103.4 ± 7.60 a	81.78 ± 5.36 a	4.49 ± 0.15 a
7 days after treatment:					
Control	32.60 ± 1.63 b	2.40 ± 0.27 b	246.0 ± 45.20 ab	82.43 ± 5.27 a	4.58 ± 0.32 a
Wind I -3.2 m/s	29.51 ± 1.26 ab	2.21 ± 0.15 ab	282.0 ± 17.40 b	95.11 ± 2.72 a	5.05 ± 0.14 a
Wind II -4.2 m/s	27.96 ± 0.57 a	1.78 ± 0.11 a	181.0 ± 15.40 a	115.56 ± 6.27 b	6.28 ± 0.52 b
14 days after treatment:					
Control	44.25 ± 2.17 a	5.00 ± 0.30 a	546.0 ± 14.0 a	89.53 ± 20.22 a	5.90 ± 1.00 a
Wind II -4.2 m/s	36.50 ± 1.34 b	3.37 ± 0.27 b	534.0 ± 57.0 a	119.33 ± 14.90 a	6.98 ± 0.39 a

1) Means within columns not followed by any letters in common are significantly different at the 0.05 probability level based on a Duncan's multiple range.

et al. 1979). Higher peroxidase activity and reduced internode elongation were observed following two days of treatment. The delay of high peroxidase activity compared to Boyer et al. results in the wind experiment here, may be attributed to the low stress level of the treatment conditions examined. This observation is supported by the results of the lower wind treatment (3.2 m/s) which yielded no significant effects (Table 17).

Peroxidase elevation by various stresses, such as mechanical injury and disease infection have been reported (Birecka and Garraway 1978). Under severe plant stress conditions such as mechanical injury, fungus attack, and disease infection, ethylene increase (Imaseki 1970), lignin formation (Vance et al. 1976) and necrotic tissues (Jaffe 1975) were observed along with increased peroxidase activity. The stress studied here, however, was more mild than these stresses and wind treated plants did not exhibit visible morphological changes except for reduced plant height. Severe wind stress conditions are expected to result in similar responses to those noted above for other severe plant stresses.

Experiments to clarify the mechanism of peroxidase increase by wind stress were not attempted here, however, studies by Jaffe (1980) suggested that growth regulators, e.g. ethylene and IAA, are involved in growth retardation and induction of peroxidases. Stimulation of peroxidases by exogenous ehtylene (Ridge and Osborne 1970) and IAA (Galston et al. 1968, Dendsay and Sacher 1978) were observed in various plants. If application of these growth regulators causes the

same effect as wind treatments, the involvement of the growth regulators, e.g. ethylene, in peroxidase elevation and wind effects may be more clearly understood.

By the mild wind conditions used here peroxidase activity increased. Wind velocity 4.2 m/s is 15.1 km/h (9.4 miles/h) and is a common field condition. Yield reduction was observed in many crops at 15-20 miles/h wind (Burns 1979). In similar field environments, the peroxidase increase will be much larger than that observed in this controlled environment. Increased peroxidase may thus be one of the wind effected plant metabolism changes and appears to be involved in growth retardation and reduced yield of maize in windy locations.

VII. SUMMARY

1. A tandem duplication of Px3-6 ($\overline{SF}$) was suggested by electrophoresis under varying gel concentrations and densitometric methods.
2. Chromosomal location of Px3 was determined using gene markers o_2, sl, Tp and Pn, and waxy translocation 7-9a. The most probable location of Px3 was assigned between 96.6 and 100.32 on the long arm of Chromosome 7.
3. Partial purification of enzymes Px3 and Px7 was achieved by Con A chromatography and Blue Sepharose chromatography. Anodal and cathodal peroxidases could be separated by Con A, and Px3 and Px7 were separated by Blue Sepharose.
4. Michaelis constant (Km) of PX3 and Px7 were determined for allozymes at Px3 and Px7 loci on the substrates--o-dianisidine, caffeic acid and ferulic acid. Px7 showed higher affinity to ferulic acid, a lignin precursor. No significant difference was found in Km values among allozymes at Px3 and Px7.
5. The molecular size of Px7 was shown to be 72,000, about twice as large as Px3 (MW 35,000). No evidence was provided that Px7 is dimeric or polymeric proteins.
6. Leaf peroxidase activity increased during development (4-7 weeks) of five non GA-responding dwarf mutants--br, br2, na, na2 and py--while two GA-responding dwarfs, d and d-tn had the same level of peroxidase activity as control.

7. Other types of morphological mutants, Kn, sl and Rg also showed high peroxidase activity relative to control. Increasing peroxidase activity in mutant Rg was characterized by an isozyme previously unreported and designated Px-Rg.
8. By wind treatment (4.2m/s), retardation in plant growth (plant height and fresh weight) and elevated peroxidase activity were observed.

APPENDIX

Appendix 1. Segregation of progenies in several inbred lines[1] of maize.

Cross		Plants with Phenotype S	SF	F	X^2	P
F_2:[2]	AA8 X 442a self	21	30	22	1.999	.25 - .50
	AA8 X 190a self	49	83	31	1.414	.25 - .50
	AA14 X 6053 self	14	25	14	0.856	.50 - .75
Backcross:[3]						
	(AA8 X P51) X AA8	27	15	-	3.730	.05 - .10
	(AA7 X 661) X 661	-	14	6	3.200	.05 - .10
	(AA6 X C42) X C42	-	13	10	0.392	.50 - .70

1) AA6, AA7, AA8, AA14--Px3-1(S)
442a, 190a, 6053, P51, C42, 661--Px3-2(F)
2) Expected ratio 3:1
3) Expected ratio 3:1

Appendix 2. Occurrence of Px3 alleles in inbred lines of *Zea mays* L.

Px3 Alleles and Type of Corn	Inbred Lines
Px3-1 (S)	
sweet corn	245, 381a, 303b, 5125, 5446, 5490, AA4, AA5, AA6, AA7, AA8, AA9, AA10, AA11, AA12, AA13, AA14, AA15, AA23, P39-5, T19, T36
field corn	A239, A509, A619, A635, Ant2(Hi34), B14a(Hi25), B37, B68(Hi31), B77, CI21E(Hi26), CI66, CM105(Hi28), CM111(Hi29), F6, GE54, H49, H55, H84, H94, H95, K55, Ky21, KyS, M14, Mo1W, Mo5, Mo17, N28, ND203, Oh07, Oh43, Oh45, Oh51a, Oh545(Hi32), R168, Tx601, W64A, WF9
Px3-2 (F)	
sweet corn	190a, 304A, 382-11, 442a, 647, 650, 661, 2132(sh2), 2256(sh2), 6053, AA1, AA2, AA3, AA16, AA17, AA19, AA21, AA22, P39, T23, T55
field corn	A226, A295, B70, C42, C103, C123, C164, H60, Hi27, L289, M822a, NC7, NC236, T115, Tx303

Appendix 3. Occurrence of Px3 alleles in tropical races and composites of Zea mays L.

Px3 Alleles	Tropical Races
Px3-1 (S)	Conico
Px3-2 (F)	Argentine pop, Avati tupi, Cabuyo, Sabanero
Segregated (S, F, S/F)	Antigua GR2, Amagaceno, Andaqui blanco, Cacao, Celaya, Chandelle, Chapalote, Chococeno, Chococeno blanco, Comiteco, Comun amarillo, Costeno amarillo, Cuban Flint, Dzit-Bacal, Early Caribbean, Guam corn, Guirua mezcla, Harinoso de Ocho, Haitian Yellow, Inbricado blanco, Maize dulce, Montana, Nal-Tel (428), Negrito, Olotillo, Pepetilla, Reventador, RH507, Salvadoreno 142, St. Croix 298, Tepecintle 70, Tuxpeno, UPCA Var 1, UPCA Var 2, UPCA Var 3, UPCA Var 4, Vandeno 251, V520C, Yucatan (mezcla), Zapalote chico, Zapalote grande, Teosinte.

Appendix 4. Px3-3(S*) allele: (Brewbaker unpublished data)

a) Progeny tests of Px3-3 (S*) allele.

Cross	Plants with Phenotype S	S*	F/S*	F	S/F	Expected Phenotypic Ratio
S*/F X S*/F	0	8	29	8	0	1:2:1 X^2=3.76 P= .10-.20
F/F X S*/F	0	0	18	17	0	FS*:F = 1:1 X^2=0.03 P= .70-.90
S/F X S*/F	2	0	4	6	4	S:F:SF:S*F = 1:1:1:1 X^2=2.00 P= .50-.60

b) Segregation of Px3-3 (S*) allele in the tropical races.

Races	Genotype S	SF	F	S*	SS*	S*F
Clavo A	8	23	44	0	0	0
Clavo B	7	15	13	0	0	0
Clavo C*[1)]	12	51	43	3	4	8

1) Gene frequency in Clavo C*: Px3-1(S) .326
Px3-2(F) .599
Px3-3(S*) .074

Appendix 5. Px3-4(F*) allele: (Brewbaker unpublished data)

a) Progeny tests of Px3-4 (F*) allele.

Cross	Plants with Phenotype S	SF	F	SF*	FF*	Expected Ratio	
S/S X S/F*	24	0	0	42	0	S:SF* = 1:1	X^2=4.90 P= .05-.025
S/F X S/F*	11	6	0	5	8	S:SF:SF*:FF* = 1:1:1:1	X^2=3.63 P= .30-.50

b) Segregation of Px3-4 (F*) allele in the tropical races of maize.

Races	Genotype S	SF	F	SF*	FF*	Total
Puya 2	14	25	8	3	0	50
Puya 13	0	2	13	2	1	18
	14	27	21	5	1	68

Gene frequency: Px3-1(S) = .441
Px3-2(F) = .515
Px3-4(F*) = .044

X^2 = 4.63 P = .10 - .50

Appendix 6. Segregation of Px3-5(F**) and Px3-6(SF) in the tropical races, Puya 10 and Puya 11 (Brewbaker, unpublished data).

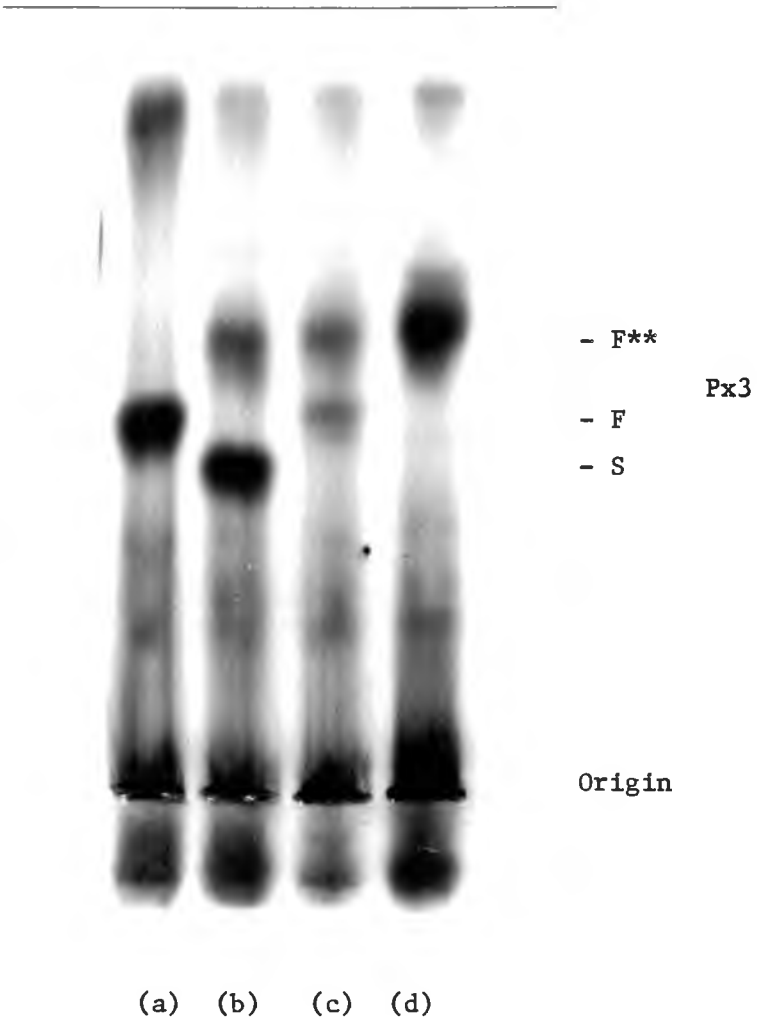
Race	Genotype							
	S	SF[1)]	F	F**	SF**	FF**	SFF**	Total
Puya 10	7	47	16	0	6	6	3	
Puya 11	6	36	24	2	8	5	6	
Total	13	83	40	2	14	11	9	172

1) including SF/SF, SF/F, S/F, S/SF.
2) Gene frequency: Px3-2 = .385
Px3-1 = .237
Px3-5 = .1105
Px3-6 = .267

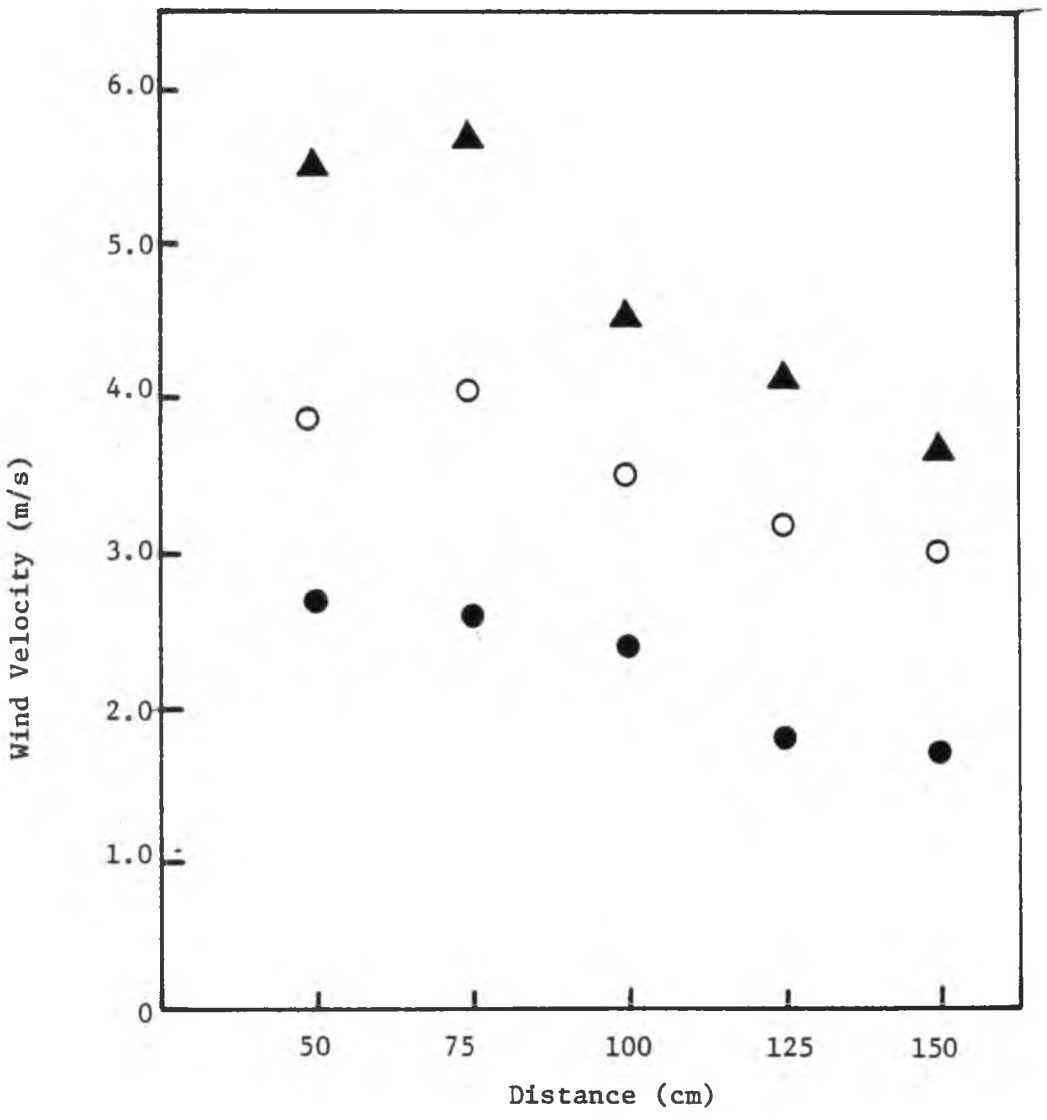
$X^2 = 7.45$ P = .10 - .50

Appendix 7. Progeny tests of Px3-5 and Px3-6 alleles.

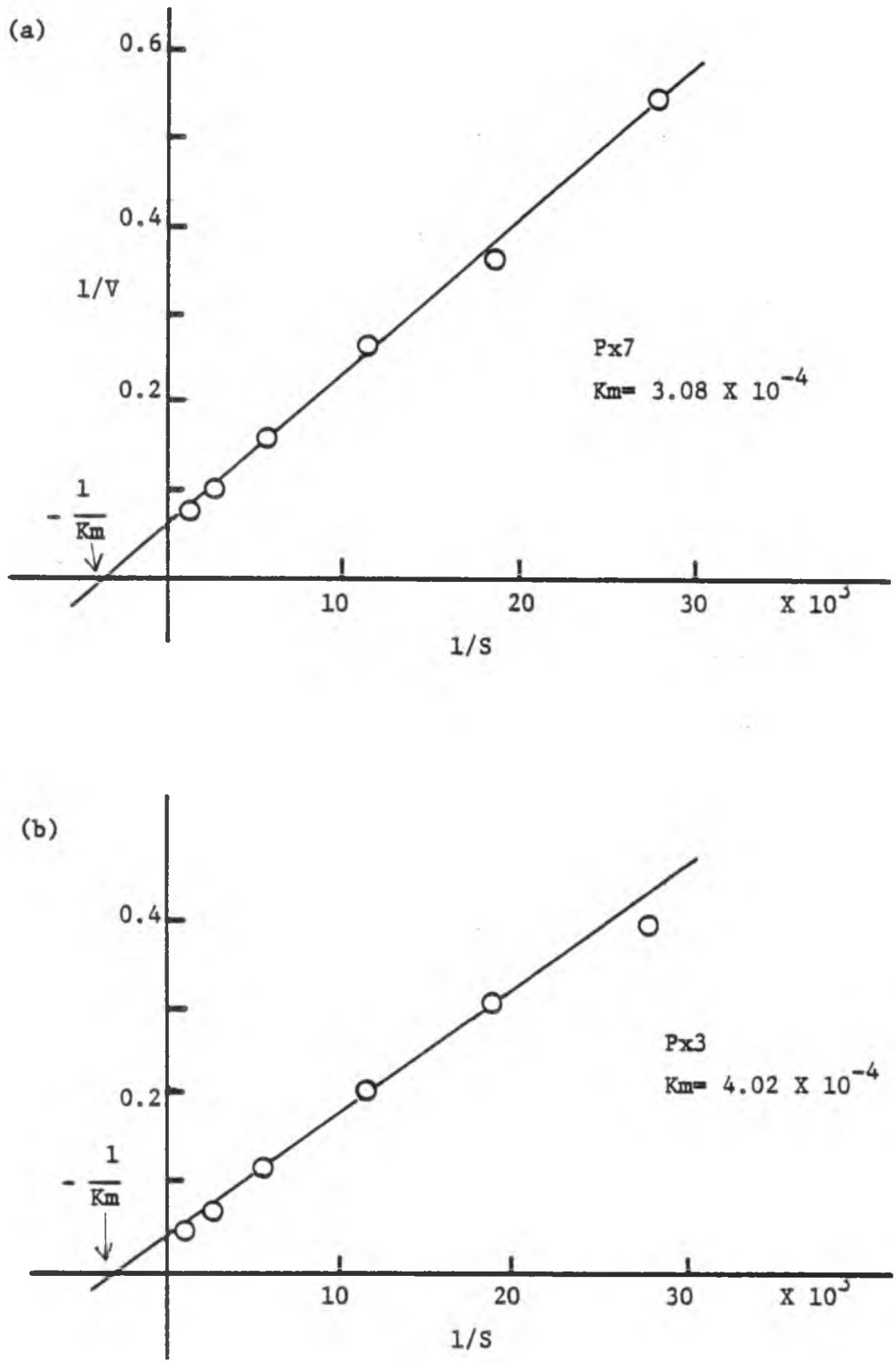
Cross	Plants with Phenotype							
	S/F**	F/F**	F**/F**	$\overline{SF}$/F**	S/F	S/S	F/F	
F**/F** X F**/F**	0	0	38	0	0	0	0	
F/F** self	0	45	17	0	0	0	20	X^2=1.0, P= .50 - .70
S/F** self	36	0	16	0	0	4	0	X^2=9.728*
F/F** X F/F	0	88	0	0	0	0	92	X^2=.088, P= .70 - .90
F/F** X S/S	188	0	0	0	161	0	0	X^2=2.01, P= .10 - .20



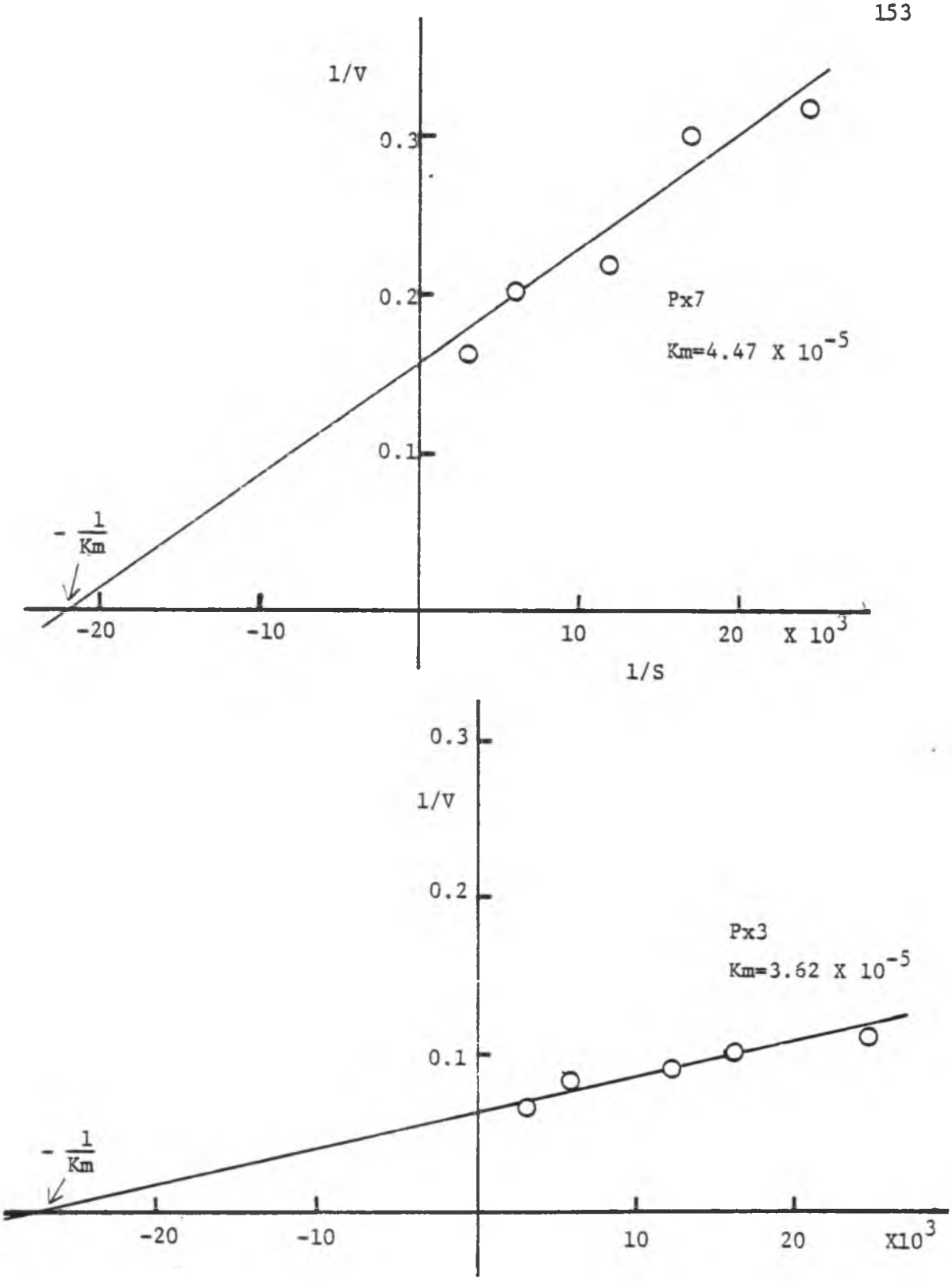
Appendix 8. Zymogram of genotypes of Px3-5(F**) alleles: (a) F/F(check), (b) S/F**, (c) F/F** and (d) F**/F**.



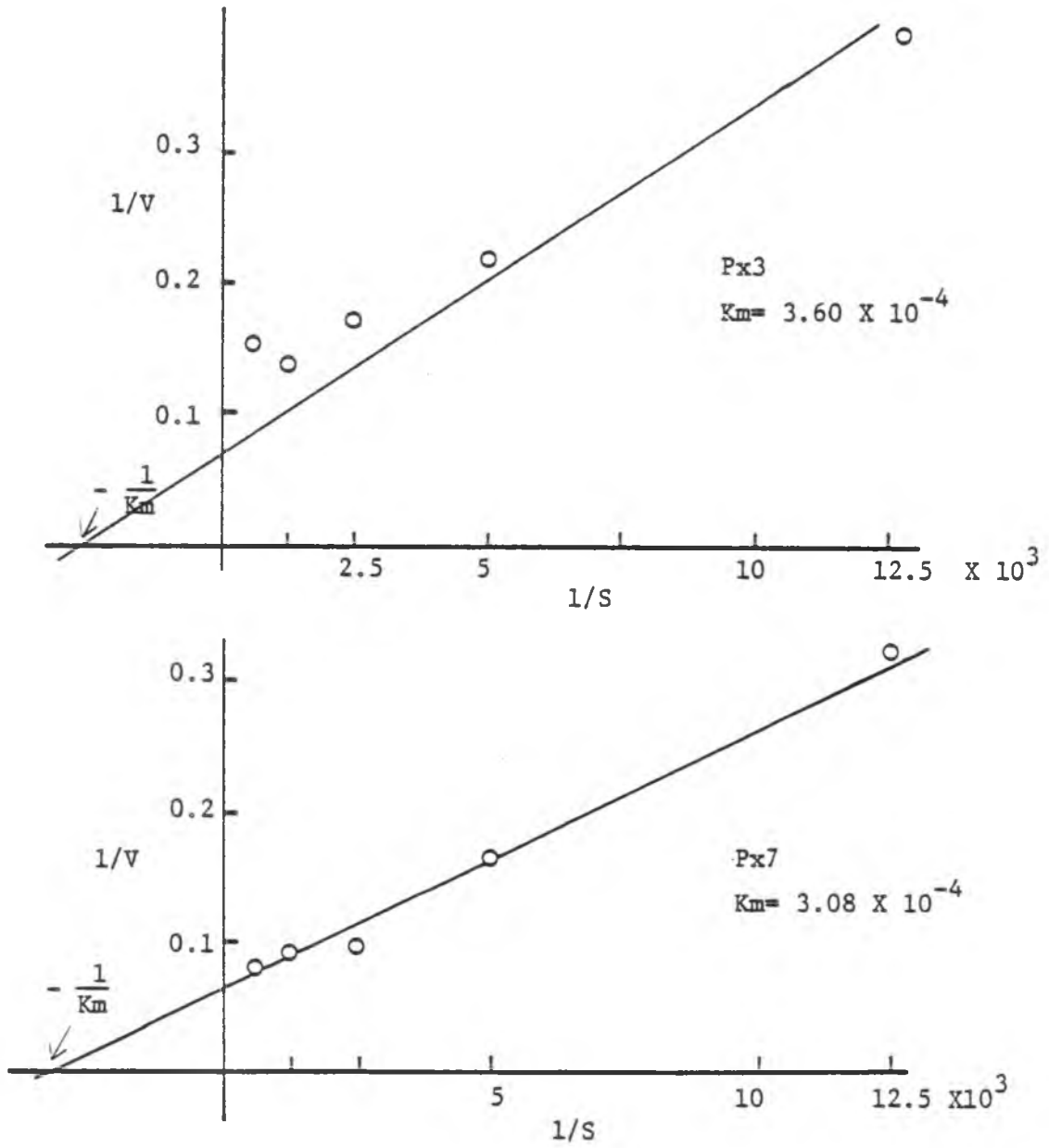
Appendix 9. Mean velocity of wind created from the electric fans (Toshiba 16"" osillating fan). Velocity I (●), II (○), and III (▲).



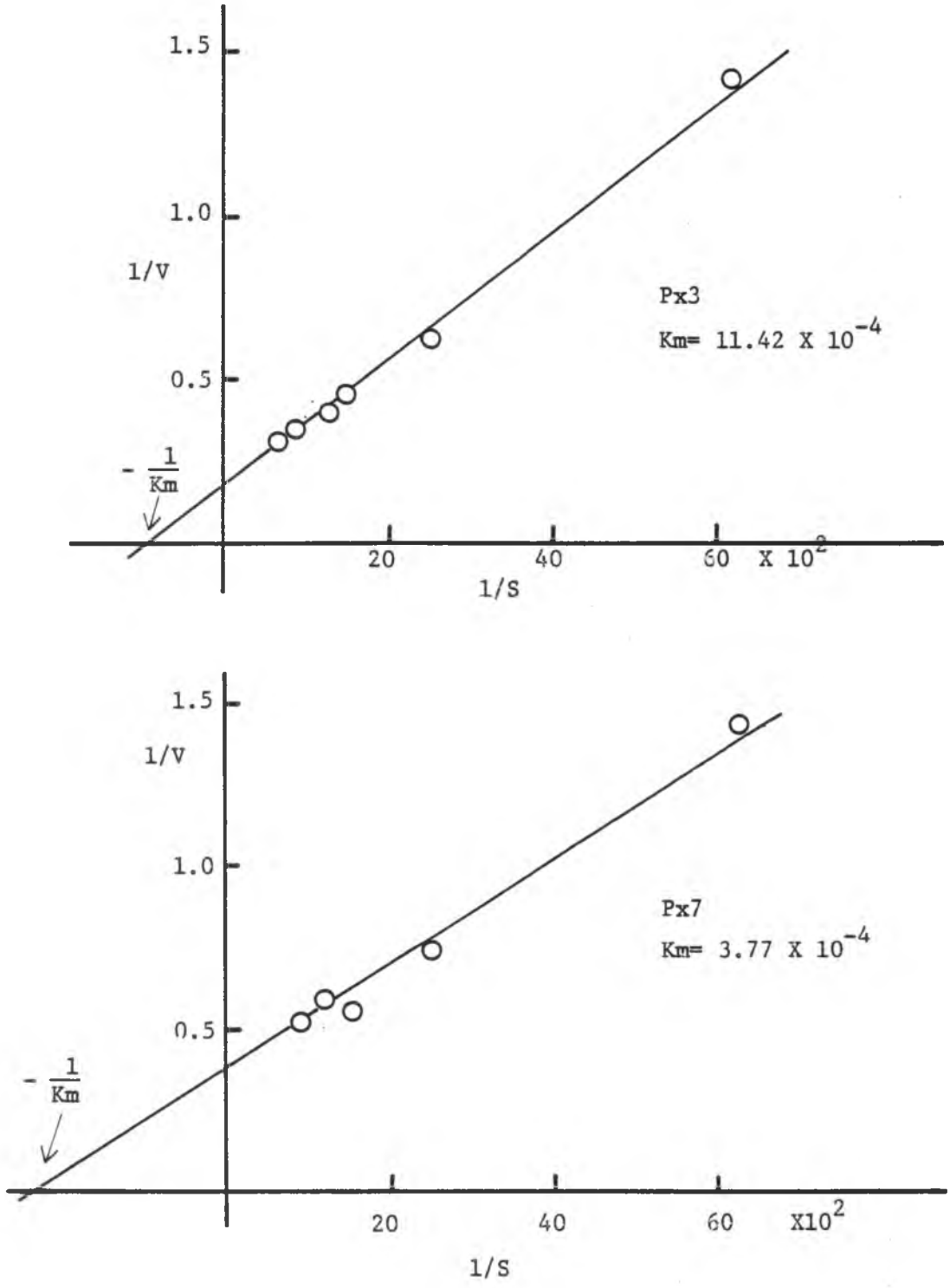
Appendix 10. Determination of Km for H_2O_2. (a) Px7-S, (b) Px3-F.



Appendix 11. Determination of Km for o-dianisidine. (a) Px7-S, (b) Px3-F.



Appendix 12. Determination of Km for caffeic acid. (a) Px7-S, (b) Px3-F.



Appendix 13. Determination of Km for ferulic acid. (a)Px7-S, (b)Px7-F.

Appendix 14. Km determinations of peroxidases to H_2O_2.

Line	Px3	Km	Px7	Km
		-- M --		-- M --
Hi27	F/F	4.02×10^{-4}	S/S	3.08×10^{-4}
B37	S/S	3.09×10^{-4}	---	---
F**	F**/F**	3.19×10^{-4}	---	---
$\overline{SF}$	$\overline{SF}/\overline{SF}$	3.97×10^{-4}	---	---
	mean:	4.00×10^{-4}		

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